Direct Reprogramming of Fibroblasts into Smooth Muscle Cells

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Direct Reprogramming of Fibroblasts into Smooth Muscle Cells

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Dedicated to my parents

Abstract

The generation of induced pluripotent stem (iPS) cells is a useful tool for regenerative medicine. However, the risk of tumor development of the aforementioned cells should be addressed before they can be used for clinical applications. During the reprogramming process a number of signal pathways are activated, which may lead to direct differentiation of specific cell lineages prior to the cells reaching the pluripotent state. In order to test this hypothesis we designed a combined protocol of reprogramming and differentiation in an attempt to achieve direct differentiation of fibroblasts to specific cell lineages. Human fibroblasts were shortly reprogrammed by overexpression of four reprogramming factors (OCT4, SOX2, KLF4 and c-MYC) and maintained in reprogramming media on a gelatin substrate for four days. These cells were defined as partially induced pluripotent stem (PiPS) cells. PiPS cells did not form tumours *in vivo* and differentiated into smooth muscle cells (SMCs) when seeded on a Collagen IV substrate and maintained in differentiation media (DM). The PiPS-SMCs expressed a panel of SMC markers such as SMA, SM22 and Calponin at mRNA and protein levels. Immunofluorescent staining of PiPS-SMCs showed positive staining for the above markers, demonstrating a typical SMC morphology. These cells displayed a greater potential to differentiate into SMCs than iPS cells. In order to elucidate the mechanism of PiPS cell differentiation into SMCs, data from a series of experiments indicated that the gene DKK3 was involved in SMC differentiation of PiPS cells. DKK3 was expressed in parallel with SMC markers, while its overexpression or stimulation induced SMC marker expression. Furthermore, DKK3 silencing resulted in downregulation of SMC markers on both the mRNA and protein levels.

Finally, additional experiments revealed that the upregulation of SMC markers by DKK3 is mediated by activation of Wnt signalling through interaction of DKK3 with the transmembrane receptor Kremen 1. Therefore, we developed a protocol to generate SMCs from PiPS cells through a DKK3 signal pathway. These findings provide a new insight into the mechanisms of SMC differentiation with therapeutic potential to vascular disease.

Acknowledgments

I wish to express my sincere appreciation to Professor Qingbo Xu for his support and help with the different aspects of the project, both technical and academic. I would especially like to express my gratitude to my supervisor Dr. Andriana Margariti for her invaluable advice and guidance in the design of the current PhD project and for her patience and encouragement throughout the years. Enormous thanks goes to my family and especially my parents for their daily support and faith in me. Furthermore I would like to thank Dr Lingfang Zeng, Dr. Anna Zampetaki and Dr. Athanasios Didangelos for their constructive comments and suggestions. I would also like to thank all the members of the Xu lab and in particular Dr. Saydul Alam, Dr. Mei mei Wong, Dr. Yanhua Hu, Dr Russel Simpson and Ms Elisabetta Di Bernadini for daily putting a smile on my face. I could not forget Dr. Bernhard Winkler for his help with the *ex vivo* experiments and of course Sherrie King for her moral support. Enormous thanks also goes to Dr. Jason Mellad for his love towards DKK3 and for agreeing to proof read my thesis. Finally, massive thanks goes to all the people in the Shah and Shanahan lab for making my everyday life in the lab a pleasant experience and especially Dr. Ioannis Smyrnias for his lovely coffee and Greek company. Finally, this work would not have been possible without the funding by the British Heart Foundation.

Thank you to you all ☺

Declaration

I, Eirini Karamariti, confirm that the work presented in this thesis is my own and I have been involved in the design, planning and conduct of all the experiments and the thesis writing. This includes the generation of PiPS cells, the differentiation of PiPS cells to SMCs, cloning, standard molecular biology methods and analysis of the results.

Expert assistance was provided in some aspects of the project by the following colleagues from the Cardiovascular Division of King's College.

Dr. Andriani Margariti prepared the samples for the *in vivo* implantation of iPS and PiPS cells which were then implanted by Dr. Yanhua Hu. She also performed the FACS analysis and cell sorting for the PiPS cells as part of a submitted manuscript (Margariti *et al*). Finally, she kindly generated all the lentiviral particles.

Dr Bernhard Winkler who performed all the *ex vivo* bioreactor tissue engineering experiments

Table of Contents

List of Figures

List of Tables

Abbreviations

±SEM	standard error of mean
3D	three dimensional
4D	four days
aa	amino acid
CD133	cluster of differentiation 133
CD34	34cluster of differentiation 3
cDNA	complementary deoxyribonucleic acid
cKit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
cMyc	similar to v-MYC (myelocytomatosis viral oncogene)
CoIP	Co-immunoprecipitation
CRD	cystein-rich domain
Ctl	control
DAPI	4',6-diamidino-2-phenylindole
DKK1-4	Dickkopf 1-4
DKK3	Dickkopf-related protein 3
DNM3L	DNA (cytosine-5-)-methyltransferase 3-like
EBs	embryoid bodies
ECFCs	endothelial colony forming cells
ECM	extracellular matrix
ECs	endothelial cells
ELISA	Enzyme-linked immunosorbent assay
ESCs	embryonic stem cells
EtBr	Ethidium Bromide
FACS	Fluorescence activating cell sorting
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HE	hematoxylin
hESC	Human Embryonic Stem Cells
hiPS	Human induced pluripotent stem cells
HRP	horse radish peroxidase
i.e.	id est
IDT	integrated DNA technologies
IIFA	Indirect Immunofluorescent assay
iPS cells	Induced pluripotent stem cells
KDR	Kinase insert domain receptor
KLF4	Krüppel-like family of transcription factor 4
Krm1	Kremen 1
Krm2	Kremen 2
LiCl	Lithium cloride
MEFs	mouse embryonic fibroblasts
MPZL2	Myelin protein zero like-2

mRNA	messenger ribonucleic acid
NDUFA4L2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2
NHDF	neonatal human dermal fibroblasts
NSCLC	non-small cell lung cancer
OCT4	octamer-binding transcription factor 4
PCR	polymerase chain reaction
PCR	Polymerase chain reaction
PDGF-BB	platelet derived growth factor BB
PFA	Paraformaldehyde
PiPS cells	Partial induced pluripotent stem cells
Q-PCR	quantitative real time polymerase chain reaction
REIC	reduced expression in immortalized cells
RLU	relative luciferase unit
rpm	rounds per minute
RT	reverse transcription
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
sFRP	secreted Frizzled-related protein
SGY	SOGGY
shRNA	short hairpin ribonucleic acid
SM22	smooth muscle protein 22-alpha or transgelin
SMA	smooth muscle actin
SMCs	smooth muscle cells
SMMHC	smooth muscle myosin heavy chain
SOX2	also known as SRY (Sex determining region Y)
SRF	serum response factor
STMN2	stathmin-like 2
TAE	Tris-base Acetic acid EDTA
TEK	tyrosine kinase, endothelial
TEPCs	thymic epithelial progenitor cells
TEPCs	thymic epithelial progenitor cells
TEVG	tissue engineered vascular graft
TEVG	tissue engineered vascular grafts
TGFβi	Transforming growth factor β-induced
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TU	Titration unit
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
vWF	von Willebrand factor
WIF-1	Wnt inhibitory factor 1
μg	micrograms
μl	microliters

Chapter 1
Introduction

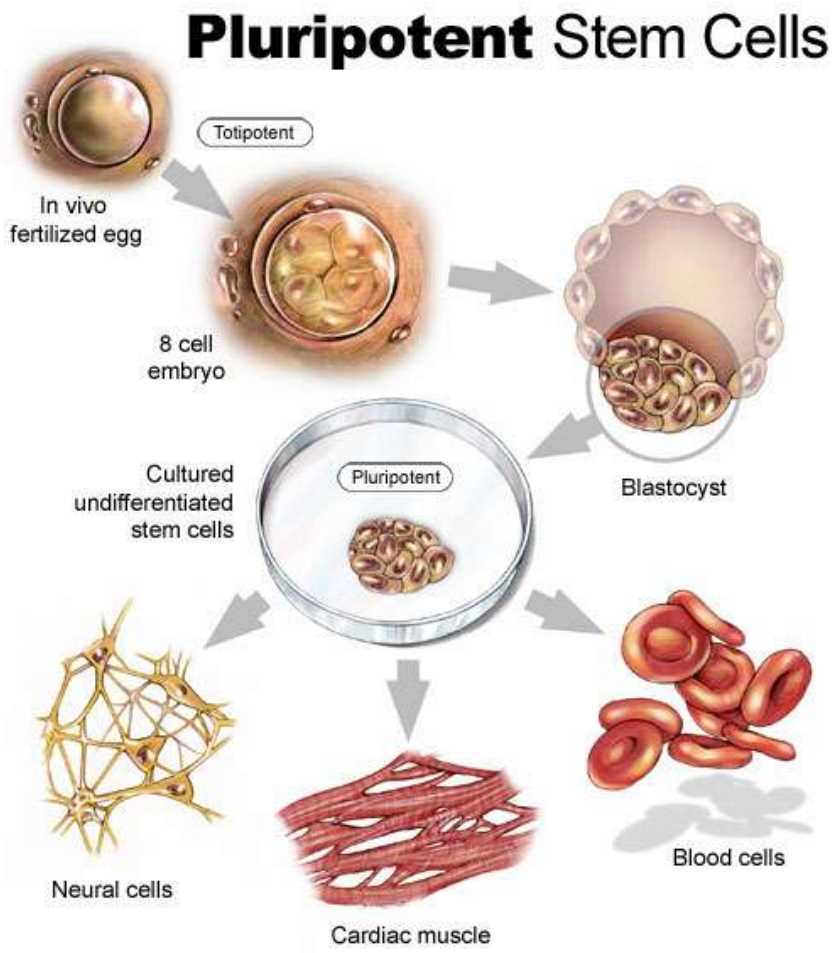
Although stem cell research is a specialised field of developmental biology for the past 140 years, it was not until recently, and due to the advent of interest in cellular therapy for degenerative diseases, that stem cell research has accelerated considerably. Their potential in recapitulating developmental paths to repair damaged tissues has made them invaluable tools in drug discovery, generation of transgenic animals and regenerative medicine (Klimanskaya et al., 2008) . However, our understanding of stem cell biology has been revolutionary as well as controversial. The limited success rate of stem cell therapy in clinical trials, with the exception of hematopoietic stem cell applications (Burt et al., 2008), has raised the possibility that stem cells require "adjuvant" molecules as enhancers during administration for tissue repair and regeneration, rendering the combination of ESC-based, high throughput screening and 3D tissue engineering approaches vital for the generation of the next tools in preclinical drug discovery (Nirmalanandhan and Sittampalam, 2009). The need for pluripotency has however lead to the expansion of stem cell research into new fields, such as the isolation of adult stem cells and the generation of induced pluripotent stem cells, opening up exciting new areas of research which hold great promise for applications in medicine and translational therapy.

1.1 Embryonic stem cells

Embryonic stem cells (ESC) are unspecialized (pluripotent) cells deriving from the inner cell mass of a blastocyst, an early stage embryo that contains 200 to 250 cells (Evans and Kaufman, 1981). Their pluripotent ability allows them to differentiate into any cell of the three germ layers (mesoderm, ectoderm, and endoderm). During development and in response to different stimuli, they give rise to specialized cells that will carry out specific functions of the body (Kleinsmith and Pierce, 1964). Although pluripotency is a key defining feature, ESCs also have another unique characteristic. Their self renewal properties give them the ability to be maintained as pure populations of undifferentiated cells in culture for extended periods of time, retaining normal karyotypes unlike tumor cell lines (Keller, 2005) (Figure 1). Over the past two decades, the different ESC lines have provided a model system for the study of the basic processes of early development and cellular differentiation making regenerative medicine and tissue engineering a real possibility for the future treatment of human disease.

During the past few years, ESCs have become a major focus of translational medicine and a reference model for understanding key molecular mechanisms which control cell fate choice and organ differentiation (Teo and Vallier, 2010). Their importance has also been captured in regenerative medicine (Ehnert et al., 2009) as well as functional tissue engineering (Guilak et al., 2001), making it possible to screen millions of small-molecule compounds available in industrial and academic centers using human primary cell lineages and “tissue constructs” or 3D cellular spheroids derived from stem cells (Nirmalanandhan and Sittampalam, 2009).

Future clinical applications of ESCs concern a broad number of degenerative diseases which could potentially be treated using ESC-based therapy such as major metabolic diseases, diverse brain and myelin disorders, heart disease and a number of other pathologies (Teo and Vallier, 2010).



http://www.csa.com/discoveryguides/stemcell/ov

Figure 1. Stem cell isolation, culture and differentiation. ESCs are isolated from the inner cell mass of early embryos and are then expanded and differentiated *in vitro* towards a variety of cell lineages upon established differentiation protocols.

1.2 ESC specific networks

Scientific research has been focused on a number of genes that are considered to be stem cell specific. Transcription factors such as OCT4 (Nichols et al., 1998), SOX2 (Avilion et al., 2003), and NANOG (Chambers et al., 2003) are responsible for maintaining pluripotency in early embryos and ESCs. Furthermore, genes like cMYC (Cartwright et al., 2005) and KLF4 (Li et al., 2005) are frequently up-regulated in tumors and have been shown to contribute to the long time maintenance of ESCs. OCT4, SOX2 and NANOG are considered to be key components in ESC pluripotency as their inactivation leads to differentiation of the cell. Through a cooperative interaction, SOX2 and OCT4 can drive pluripotent-specific expression of a number of genes including NANOG. OCT4 and SOX2 interact with the NANOG promoter which contains a highly conserved SOX-OCT cis-regulatory element essential for NANOG transcription and therefore pluripotency (Rodda et al., 2005).

Moreover, these transcription factors are essential for repressing a key set of genes needed for the development of the embryo and differentiation into different specialized cells and tissues (Figure 2). Therefore, OCT4, SOX2, and NANOG are master regulators, silencing genes that are waiting to create the next generation of cells (Boyer et al., 2005).

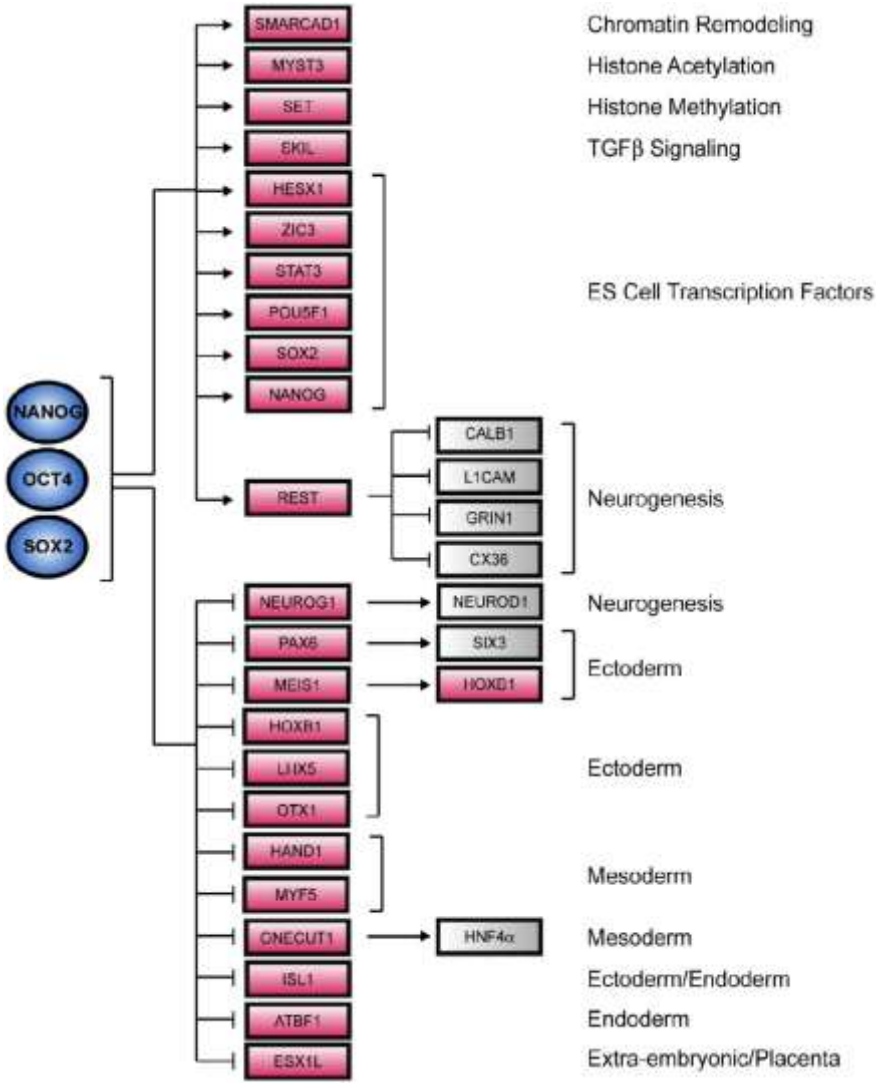


Figure 2. The core transcriptional regulatory network in ESCs. This model identifies OCT4, SOX2, and NANOG target genes that encode transcription factors and chromatin regulators as well as integrating knowledge of the functions of these downstream regulators based on comparison to multiple expression datasets and to the literature (Boyer et al., 2005).

1.3 Limitations in ESC research

ESC research has raised a number of ethical questions, as the isolation of human ESCs requires the destruction of the embryo.

Furthermore, the issue of immune system rejection still stands due to the different genetic makeup between the patient and donor. Therefore, even after perfecting the differentiation process from the pluripotent stage to that of the desired cell type, the use of powerful anti-rejection drugs with serious side effects would be required to avoid immune rejection.

Finally, teratoma formation, which is the creation of a non-cancerous tumour formed by the accumulation of undifferentiated cells, can cause a number of problems especially in cases of syngenic ESCs (cells with same genetic makeup as the host) where immune rejection does not protect the recipient (Nussbaum et al., 2007).

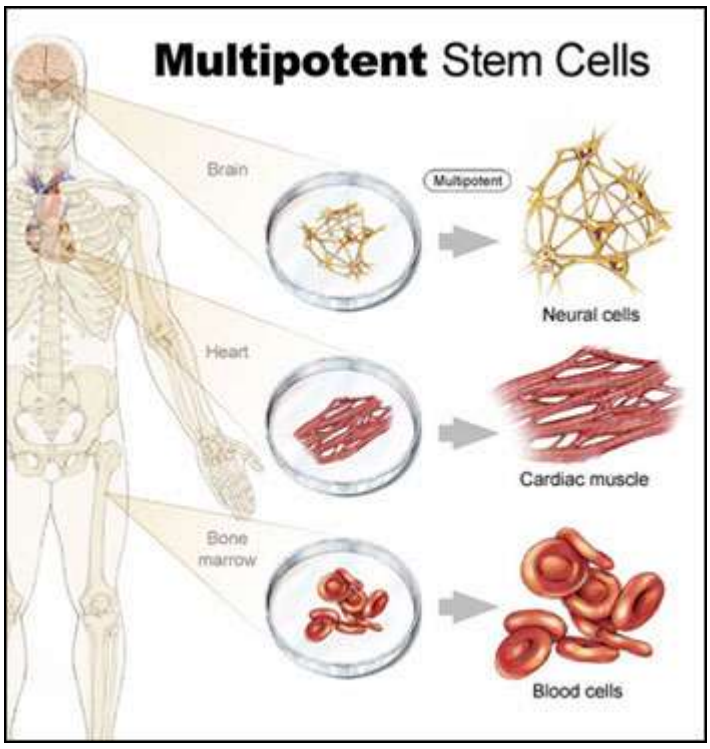
In an attempt to find a solution to the obstacles concerning the use of ESCs, scientists focused their research into finding alternative sources of pluripotent cells, ultimately generating induced pluripotent stem cells which will be discussed in a following section.

1.4 Adult Stem Cells

Adult stem cells or somatic stem cells are multipotent stem cells which originate from differentiated organs and can reside in specific niches within the adult body that are essential to maintain their property of self-renewal and differentiation (Yin and Li, 2006). The identification of such cells started about 40 years ago when hematopoietic stem cells and bone marrow stem cells (also known as mesenchymal stem cells) were identified by scientists and shown to be able to differentiate into all cellular lineages in blood and bone respectively, exhibiting multipotent potential (Nirmalanandhan and Sittampalam, 2009). Despite the great disbelief concerning the presence of multipotent stem cells within the adult tissues as recently as 20 years ago, it is now well established that adult stem cells are present in many adult tissues, including the adventitia (Hu et al., 2004), brain (Doetsch, 2003), skin (Bickenbach and Grinnell, 2004), muscle (Martin et al., 2006), liver (Kung and Forbes, 2009), heart (Beltrami et al., 2003) and lung (Neuringer and Randell, 2006) in addition to the bone marrow (Wilson and Trumpp, 2006). Adult stem cells have been shown to self renew and usually differentiate into all the cell types constituting their organ of origin as well as other lineages in a limited fashion, and are involved in the continuing maintenance and repair of the tissues and organs throughout the life span of the individual (Nirmalanandhan and Sittampalam, 2009; Teo and Vallier, 2010).

Although adult stem cells represent an ideal target for regenerative medicine, since immunosuppressive treatments would not be required, their clinical use is currently limited for diverse reasons. Apart from their limited differentiation potential, their presence in several tissues still remains controversial, making their identification and characterization a challenging task (Teo and Vallier, 2010).

Furthermore, the results obtained in animal models might not be directly translated to humans, thus limiting the study of the mechanisms controlling the self-renewal and differentiation of adult stem cells, which is necessary for the development of future clinical treatments. Somatic stem cells could also be targeted by the disease itself, explaining why they appear to be incapable or insufficient in stopping the progression of the disease (Teo and Vallier, 2010). Finally, technical issues such as the loss of proliferative capacity and differentiation potential under standard culture conditions as well as the method for the delivery of adult stem cells to the patient, comprise limitations for their clinical applications (Sekiya et al., 2002). Recent studies however, have shown that such technical issues could be circumvented by induction of somatic stem cell mobilization and recruitment in injured tissues in response to specific stimulus. More specifically, it has been demonstrated that hematopoietic progenitor cells can be mobilized from the bone marrow upon stimulation with G-CSF (Levesque et al., 2003) and that discrete populations of progenitor cells can be differentially mobilized from the bone marrow as a response to different factors, suggesting selective recruitment of progenitor populations in different pathologies (Pitchford et al., 2009). Additionally, it has recently been demonstrated that a progenitor population of epicardial origin can give rise to *de novo* cardiomyocytes under the circumstances of a myocardial infarction and that the peptide thymosin β4 can enhance this process, thus suggesting a regenerative mechanism within the mammalian adult heart (Smart et al., 2011). Further studies in the mobilization of specific subpopulations of progenitor cells for tissue regeneration could prove extremely useful in the design of non invasive therapeutic strategies.



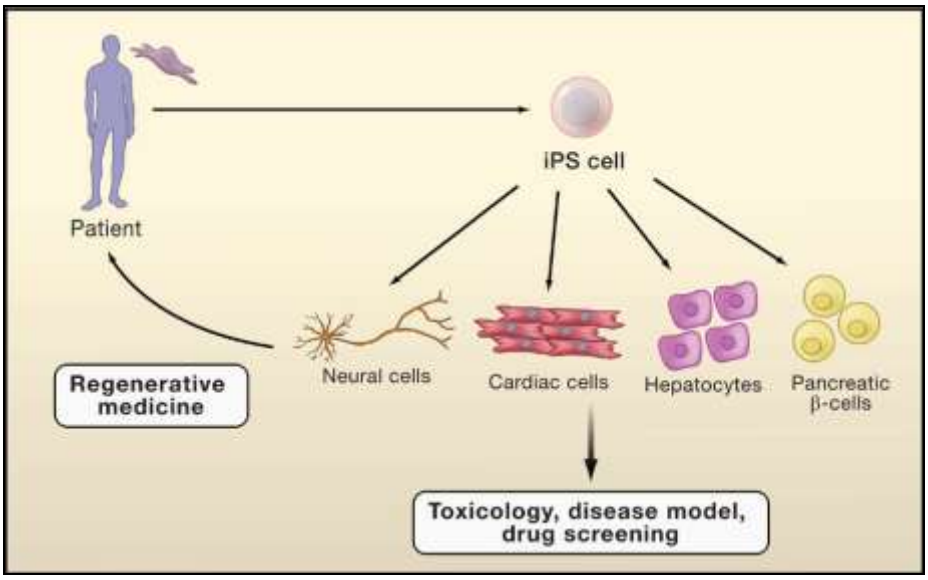
http://www.kokhucredernegi.org.tr/eng/kok_hucre/eriskin.html

Figure 3. Niches of adult stem cells. Adult stem cells have successfully been isolated from a variety of tissues such as bone marrow, the heart and the brain and differentiated towards all cell types constituting their organ of origin.

1.5 Induced pluripotent stem cells (iPS cells)

One of the newest and most exciting areas of stem cell research is the development of iPS cells. iPS cells are pluripotent cells artificially derived from terminally differentiated somatic cells through overexpression of four genes.

The ethical and immunological problems often associated with the use of ESCs are bypassed as the origin of the iPS cells can be a somatic cell population isolated from the patient (such as dermal fibroblasts). The potential of these cells is enormous, including their application in effective and safe drug screening, regenerative medicine as well as their contribution in constructing disease models (Figure 4).



(Yamanaka, 2009)

Figure 4. Applications of the iPS cell technology. iPS cells can produce various somatic cells with the same genetic information as the patient, which could be used to construct disease models, screen effective and safe drugs and treat patients through cell transplantation therapy.

1.5.1 Generation of iPS cells

Since 1997, it was already known that somatic cells can be reprogrammed into a pluripotent state by transferring their nuclear contents into oocytes (Wilmut et al., 1997) or by fusion with ESCs (Cowan et al., 2005), indicating that factors exist within the unfertilized eggs which are able to confer totipotency or pluripotency to somatic cells (Tada et al., 2001).

Taking advantage of this knowledge, in 2006, Takahashi and Yamanaka, in a landmark paper, demonstrated that terminally differentiated mouse fibroblasts in culture, could be reprogrammed into pluripotent ESC like cells by retroviral transduction of four defined transcription factors, SOX2, OCT4, KLF4 and cMYC (Takahashi and Yamanaka, 2006). These cells were named iPS cells and exhibited similarities with ESCs in cell morphology, proliferation, expression of some ESC genes and the ability to form teratomas. DNA demethylation of the promoter regions of OCT4 and NANOG was also observed indicating re-activation of endogenous genes associated with pluripotency. In addition, the somatically silent X chromosome was re-activated in female cells (Hochedlinger and Plath, 2009). However, differences were observed between their global gene expression pattern and that of ESCs. Furthermore, they failed to produce adult chimeric mice.

One year later, further characterization identified a method to generate mouse iPS cells with phenotypes comparable to ESCs, including the ability to generate chimeric mice and observe germline transition (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007).

1.5.2 Generation of human iPS cells

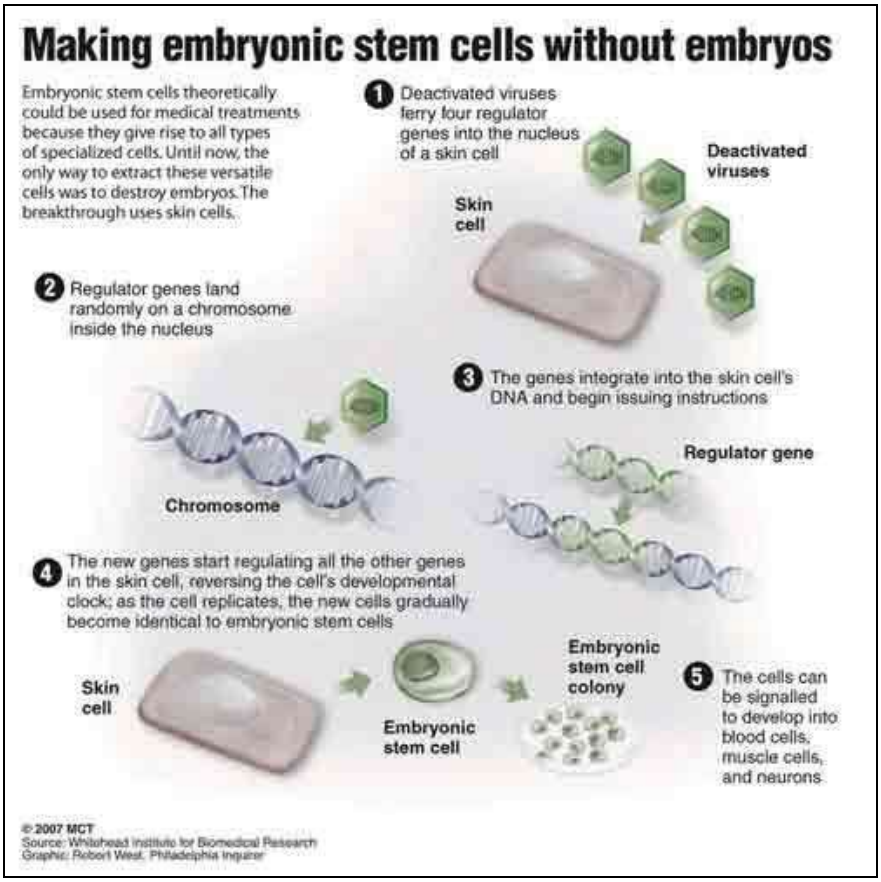
In November 2007, two separate teams generated iPS from human skin cells that were phenotypically indistinguishable from human embryonic stem cells, a milestone in the field of disease modelling and patient specific regenerative medicine.

Shinya Yamanaka at the University of Kyoto showed that terminally differentiated human fibroblasts can be reprogrammed to an embryonic like state using the same formula previously used to reprogram mouse fibroblasts (retroviral transduction of SOX2, OCT4, KLF4,cMYC) (Takahashi et al., 2007). These cells could differentiate into any of the three cell types (Figure 5).

Simultaneously, Thomson and Yu published the success of a similar approach. Starting from a panel of 14 genes known to be expressed in human ESCs and through the process of elimination, they generated pluripotent cells from terminally differentiated cells using a different combination to the one used by Yamanaka in that KLF4 and cMYC were replaced with NANOG and LIN28. These cells expressed genes and surface proteins characteristic of ESCs and were capable of teratomas formation or tumors with cells representing the three embryonic germ layers (Yu, Vodyanik et al. 2007).

As it was proven later on, this method of cellular reprogramming is not specific only to mouse or human fibroblasts, but can be applied in different species and kinds of terminally differentiated cells such as lymphocytes (Hanna et al., 2008), liver cells (Aoi et al., 2008), intestinal cells (Wernig et al., 2007) and neural progenitors (Eminli et al., 2008). In addition iPS cells have been generated from both monkey (Liu et al., 2008)

and rat (Liao et al., 2009) proving in vitro reprogramming to be a universal process. However, an alteration in the combination of factors might be required for successful reprogramming of different cell types depending on the endogenous expression pattern within those populations.



http://ncronline.org/node/31

Figure 5. Generation of iPS cells. Schematic representation of a standard protocol for iPS cell generation. Skin cells are infected with viruses carrying a cocktail of reprogramming factors which integrate into the genome and alter the genetic program of the cells from terminally differentiated to pluripotent cells.

1.5.3 iPS cell application challenges

Even though iPS cell technology could potentially be the solution to obstacles concerning the use of ESCs, clinical application of the aforementioned cells faces many obstacles itself.

Teratoma formation is one of the most prominent and common issues concerning the use of iPS cells. Even small numbers of undifferentiated cells can result in the formation of teratomas (Yamanaka, 2009). In addition, iPS cells derive from somatic cells which as we discussed, were subjected to forced reprogramming. Aberrant reprogramming might result to either an impaired ability to differentiate or might even increase the risk of immature teratoma formation after directed differentiation (Figure 6).

In most cases, iPS cells are generated by transduction of the four genes by retroviruses or lentiviruses. By nature these transgenes can be integrated into the genome disrupting the normal gene expression. Unfortunately, this integration usually falls into an oncogene or tumor suppressor loci leading to malignant transformation(Cohen and Krause, 2009). The transgenes, although mostly silenced in iPS cells, can be reactivated and lead to tumorigenesis (especially cMyc, which is a known oncogene linked with tumorigenesis) (Okita et al., 2007).

Another concern that was recently raised concerning the therapeutic application of iPS was their immunogenicity. While iPS cells were generally assumed to be immune-tolerated by the recipient from whom they derived, it was demonstrated that in contrast to the derivatives of ESCs, abnormal expression in some cells derived from iPS cells can induce T-cell-dependent immune response in syngenic recipients (Zhao et al., 2011).

Therefore it is advised that immunogenicity of these patient specific iPS cells is vigorously evaluated before their application into patients.

Finally, most of the protocols used to date demonstrate reprogramming efficiencies as low as 0.01%. The low efficiency rate may reflect the need for rare genetic and/or epigenetic changes in the original somatic cell population or in the prolonged culture. It also suggests the methods utilised to generate iPS cells need to be improved and new strategies to be developed (Hochedlinger and Plath, 2009).

1.5.4 Different methods of generating iPS cells

Since the first generation of iPS cells and in an effort to optimize the reprogramming protocols as well as to overcome the various obstacles during the process, a number of techniques of generating these cells have been established (Figure 6).

Retroviruses and lentiviruses: The standard protocol for generating iPS cells is the overexpression of any of the two combinations of four transcription factors (SOX2/OCT4/KLF4/cMYC or SOX2/OCT4/NANOG/LIN28) as part of a lentiviral or retroviral construct. The cells are infected with the four viruses and maintained in ESC medium for 1-2 weeks for mouse cells or 3-4 weeks for human cells before iPS cell colonies are picked up, expanded and characterised. However, the use of these viruses raises safety issues due to the possibility of random integration within the genome. In an attempt to minimize the amount of proviral integrations, iPS cells have also been generated from both human and mouse cells using a single polycistronic vector encoding the 4 factors simultaneously (Carey et al., 2009; Takahashi and Yamanaka, 2006; Yu et al., 2007).

Adenoviruses*:* It has been shown that iPS cells can be generated from mouse hepatocytes using adenoviruses carrying the four reprogramming factors, a technique that eliminates viral integration (Stadtfeld et al., 2008b) but occurs at an even lower efficiency than previously stated.

Plasmids: It has also been shown that iPS cells can be generated from mouse embryonic fibroblasts using large constructs containing three of the four genes within the same construct (SOX2/OCT4/KLF4). cMYC was administered in a different construct. Repeated transfection of the 2 constructs resulted in iPS cells without evidence of plasmid integration addressing a critical safety concern for potential use of iPS cells in regenerative medicine (Okita et al., 2008).

Proteins: Another interesting technique is that of generating iPS cells using recombinant proteins. It was demonstrated that iPS cells can be generated from murine embryonic fibroblasts following culture with the reprogramming proteins tagged with peptide sequences which markedly enhance protein uptake by cells. The iPS cells generated were able to self-renew and were shown to be pluripotent *in vitro* and *in vivo* (Zhou et al., 2009).

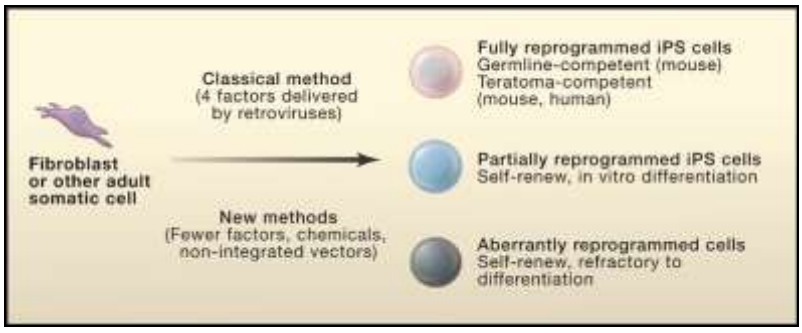
Cre-mediated excision reprogramming*:* Recently, iPS cells were generated by genomic integration of the four genes using plasmids (Kaji et al., 2009), lentiviruses (Soldner et al., 2009), or transposons (Woltjen et al., 2009) followed by transgene removal using Cre-mediated excision or re-expression of transposase (Yamanaka, 2009).

Chemicals and small molecules: iPS cells have been generated by using chemicals that can replace one or two of the four genes during cell reprogramming such as valproic acid

which can replace cMyc, and BIX-01294 and BayK8644 which can replace Sox2 (Huangfu et al., 2008; Shi et al., 2008).

Synthesised mRNA reprogramming: Another non integrating strategy for reprogramming cell fate is based on administration of synthetic mRNA modified to overcome innate antiviral responses. This approach has the ability to reprogram a variety of human cell types to pluripotency with efficiencies that surpass already established protocols and represents a safe strategy with broad applicability for disease modelling and regenerative medicine (Warren et al., 2010).

microRNA mediated reprogramming: One of the most recent strategies of generating iPS cells is the utilization of the rapidly expanding field of miRNAs. It has been shown that the expression of the miR302/367 cluster rapidly and efficiently reprograms mouse and human somatic cells to an iPS cell state without the requirement of exogenous transcription factors and with two orders of magnitude higher efficiencies that the standard transcription factor strategy (Anokye-Danso et al., 2011).



(Yamanaka, 2009)

Figure 6. Old and New Ways to Generate iPS cells. During the reprogramming and regardless of methodology, the derivatives can be fully reprogrammed cells that are comparable to ES cells, partially reprogrammed iPS cells that can self-renew and differentiate into certain cell lineages, or aberrantly reprogrammed cells that self-renew but are refractory to differentiation.

1.5.5 Epigenetic modifications and epigenetic memory in iPS cells.

As mentioned above, iPS cell generation is a lengthy and inefficient process with many cells ultimately failing to be completely reprogrammed. Although several functional assays exist to determine the developmental potential and limitations of pluripotent cells in mice (Jaenisch and Young, 2008), such assays cannot be used for human ESCs and iPS cells, making a comprehensive characterization of the epigenetic state an essential substitute (Meissner, 2010). In an attempt to elucidate the mechanisms by which ectopic transcription factors override the existing epigenetic state and change it into a specific alternative, extensive research has been focused into the complex process of epigenetic remodelling during reprogramming.

Detection of epigenetic remodelling requires comprehensive genomic characterisation which can involve gene expression profiling, chromatin state maps of key activating and repressive marks and DNA methylation analysis. Studies have shown that reprogramming somatic cells into an ESC like phenotype requires suppression of somatic genes with concomitant activation of pluripotency genes (Mikkelsen et al., 2008). Genomic analysis conducted by two separate groups has shown that successful reprogramming is dependent upon demethylation of pluripotency specific genes and methylation of promoters regulating somatic specific genes (Maherali et al., 2007; Mikkelsen et al., 2008).

Comparison of stable iPS cell clones with partially reprogrammed iPS cells revealed that although fully reprogrammed iPS cells have similar gene expression and epigenetic state to ESCs, partially reprogrammed iPS cells display hypermethylation of pluripotency loci and are closer to mouse embryonic fibroblasts (Mattout et al., 2011; Mikkelsen et al., 2008).

Finally, it has been reported that treatment with chemicals such as histone deacetylases and DNA methyltransferase inhibitors can facilitate the reprogramming and enable cells that are "trapped" in a partially de-differentiated state, to acquire an iPS cells genetic phenotype (Mikkelsen et al., 2008)

Interestingly, it has recently been reported that low passage iPS cells generated by transcription factor based reprogramming of mouse somatic cells contain residual DNA methylation signatures characteristic of their tissue of origin. These signatures favour their differentiation along lineages related to the donor cell and restrict alternative cell fates. However, such epigenetic memory could be reset by differentiation and serial reprogramming or by treatment with chromatin modifying drugs (Kim et al., 2010). Notably, Hochedlinger and colleagues observed similar epigenetic memory in iPS cells from blood, fibroblasts and muscle sources which eventually becomes extinct after continuous passaging in culture (Polo et al., 2010).

1.6 ESC and iPS cell differentiation

1.6.1 ESC differentiation

Given the proper stimulus (growth factors or substrates), pluripotent cells can spontaneously differentiate and generate various lineages in culture. Over the past few years mesoderm-derived lineages have been successfully generated from ESCs. For instance, under optimised culture conditions, following serum induction, mouse ESCs underwent hematopoietic differentiation (Keller, 1995). Furthermore, it has recently been shown that mouse ESCs plated on matrigel, but not collagen or gelatine, could form embryonic bodies and differentiate into blood vessels in the absence of additional growth factors (Nakagami et al., 2006). Vascular progenitors (Yamashita et al., 2000), cardiomyocytes (Kehat et al., 2001), endothelial cells (ECs) (Levenberg et al., 2002) as well as smooth muscle cells (SMCs) (Xiao et al., 2007) have also been generated, offering a broad spectrum of cell lineages for clinical use in transplantation and replacement therapy.

Specifically for the cardiovascular field, the generation of vascular endothelial cells, SMCs and cardiomyocytes from ESCs demonstrated a novel mechanism of cell differentiation as well as a potential source for cardiovascular tissue repair (Gepstein, 2002). Endothelial cell differentiation from ESCs has been induced within the three-dimensional tissue of embryoid bodies (EBs) by Wartenberg (Wartenberg et al., 1998). When mouse ESC-derived cardiomyocytes were generated, it was found that the aforementioned cells expressed tissue-specific markers such as structural cardiac proteins, receptors and transcription factors (Boheler et al., 2002).

Importantly, human ESCs displayed the potential to generate cardiomyocytes based on optimised protocols from murine ESCs (Mummery et al., 2003). It has also been demonstrated that $CD34^+$ cells (differentiation molecule present in mesenchymal cells) isolated from differentiated hESCs, function as vascular progenitor cells capable of producing both ECs and SMCs elucidating their developmental origin and revealing the relationship between these two cell types (Hill et al., 2010).

1.6.2 iPS cell differentiation

A variety of protocols applied in the differentiation of ESCs towards an array of different cell lineages have been used in order to differentiate iPS cells.

Taura *et al* have investigated the features of the directed differentiation of human iPS cells into vascular ECs and mural cells (MCs). Their studies have revealed that the properties of human iPS cell differentiation into vascular cells are nearly identical to those of hESCs (Taura et al., 2009b).

Additionally, Xie *et al.* have also demonstrated that iPS cells have the ability to differentiate into SMCs. In the aforementioned study, iPS cell monolayers were treated with retinoic acid for 8 days, after which they successfully expressed SMC specific markers and acquired SMC functional characteristics including contraction and calcium influx in response to stimuli (Xie et al., 2009).

In a similar study, murine iPS cells were differentiated into $Flk1^+$ progenitor cells and their mesodermal progeny, including cells of the cardiovascular and hematopoietic lineages by utilizing Collagen IV culture or EB formation (Schenke-Layland et al., 2008).

Cells of the endoderm layer have also been successfully generated by iPS cells. When iPS cells were differentiated on Collagen IV in the present of both Activin A and Lithioum Cloride (LiCl), the aforementioned cells differentiated to definitive endoderm through mesendoderm (Inami et al., 2011).Additional treatment with Fgf8 followed by Fgf7, Fgf10 and BMP4 differentiated the iPS cells to thymic epithelial progenitor cells (TEPCs) by phenotype (Inami et al., 2011).

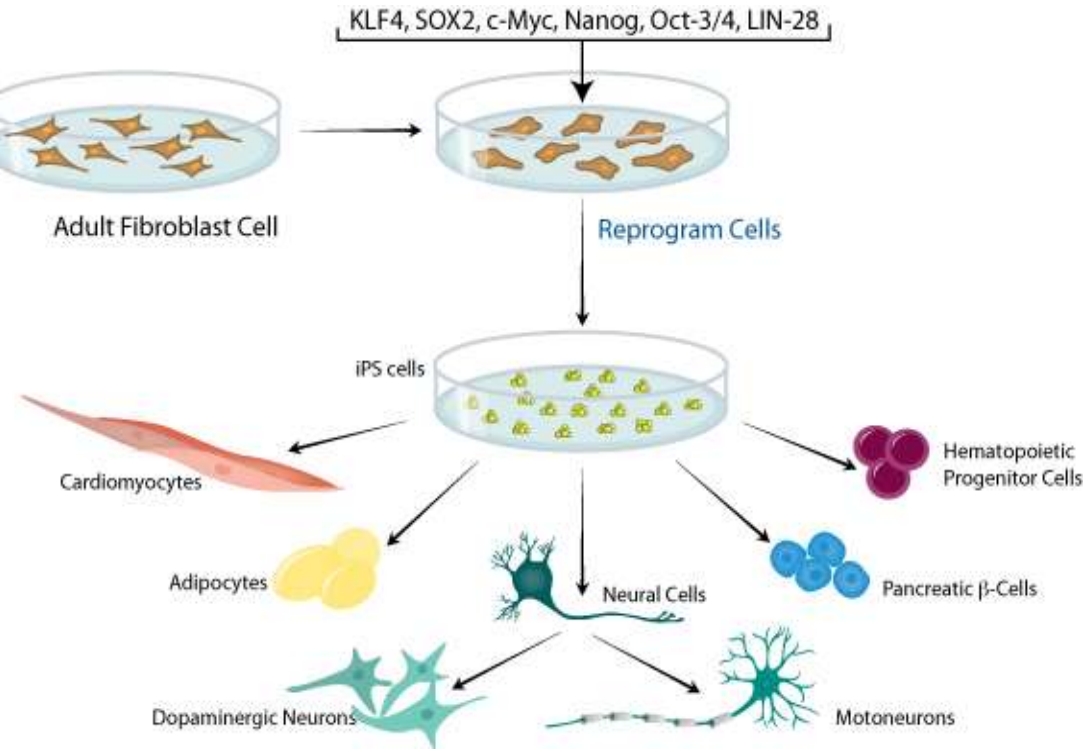
Moreover, using methods shown to be effective at generating motor neurons from human embryonic stem cells (hESCs), it was found that once induced to a neural lineage, human iPS cells could be differentiated to form motor neurons with a similar efficiency as hESCs (Karumbayaram et al., 2009).

In addition to the above studies, human iPS cell lines obtained from human fetal, neonatal, and adult fibroblasts through reprogramming were differentiated towards hematopoietic and endothelial cells using an OP9 differentiation system. Their differentiation potential was compared to that of five human ESC lines. It was then shown that although some variations in the efficiency of hematopoietic differentiation between different hiPS cells existed, the pattern of differentiation was very similar in all seven tested lines obtained through reprogramming. These findings provide strong evidence that hiPS cells are quite similar to hESCs and differentiation systems established for hESCs can be readily applied to hiPS cells (Choi et al., 2009).

Similarly, four human iPS cell lines underwent adipogenic differentiation and their properties were compared to the differentiation properties of human ESC lines. It was demonstrated that human iPS cells have an adipogenic potential comparable to human ES cells (Taura et al., 2009a).

Finally, cardiomyocytes have successfully been generated from murine iPS cells *in vitro* which exhibited functional cardiomyocyte characteristics, with established hormonal regulation pathways and expression of cardiac ion channels (Kuzmenkin et al., 2009).

In summary, iPS cells have been differentiated into a variety of cell lineages including the ones mentioned above as well as pancreatic beta-cells (Zhang et al., 2009), and several neuronal cell types (Wernig et al., 2008) paving the way for generating patient-specific pluripotent cells that could be used for research and therapy (Figure 7).



(Amabile and Meissner, 2009)

Figure 7. Direct differentiation of iPS cells. iPS cells can differentiate into a variety of cell lineages such as cardiomyocytes, neurons, and pancreatic β cells, upon specific stimulation and under established differentiation conditions.

1.7 Direct differentiation between lineages

The use of a combination of transcription factors and the generation of iPS cells caused a cascade of questions and ideas in scientists which lead to a whole new field of research. One of the latest and most exciting fields of iPS cell related research is the direct conversion of somatic cells, such as fibroblasts, into other somatic cell fates.

Although direct reprogramming, or otherwise known as lineage reprogramming, has been investigated since the early 90s, terminally differentiated cells had been limited to transforming into skeletal muscle via MyoD. This transcription factor became recognized as a ‘‘master regulator gene’’ because it was able to convert fibroblasts, chondrocytes and retinal epithelium into contracting muscle in culture (Choi et al., 1990). Additional examples included the conversion of sensory hair cells by Math1 (Izumikawa et al., 2005), B lymphocytes into macrophages by CEP/B (Laiosa et al., 2006), as well as fibroblasts into macrophage-like cells by C/EBPalpha/beta and PU.1 (Feng et al., 2008).

However, despite years of research, master regulators for other lineages still remained elusive. To answer the question of whether transcription factors could directly induce other defined somatic cell fates besides pluripotency, Ieda and colleagues showed that a combination of three developmental transcription factors, Gata4, Mef2c and Tbx5 could rapidly and efficiently reprogram postnatal cardiac or dermal fibroblasts directly into differentiated cardiomyocyte-like cells (Ieda et al., 2010)

In a similar report, Efe *et al* showed that transgenic expression of the Sox2, Oct4, Klf4, cMyc in mouse embryonic fibroblasts (MEFs) could directly reprogram fibroblasts to spontaneously contracting patches of differentiated cardiomyocytes. Furthermore, several lines of evidence suggested that a pluripotent intermediate during the transition was not involved (Efe et al., 2011).

Although all the studies mentioned above involve conversion of one cell type to another, this conversion occurs within the same lineage. In 2010, Verbuchen *et al* showed that differentiated mouse cells have the capacity to change lineage by showing direct conversion of dermal fibroblasts into neurons. Starting from a pool of nineteen candidate genes, three factors, Ascl1, Brn2 (also called Pou3f2) and Myt1l were ultimately found to be sufficient to rapidly and efficiently convert mouse embryonic and postnatal fibroblasts into functional neurons in vitro (Vierbuchen et al., 2010).

Within the same lines of work, it was recently shown that adult human fibroblasts could differentiate into functional neurons with a combination of microRNA miR-124 and thetranscription factors MYT1L and BRN2 (Ambasudhan et al., 2011) and that both mouse and human fibroblasts could be directly differentiated to functional spinal motor neurons by overexpression of select transcription factors (Son et al., 2011).

Finally, in one of the most recent studies, Marro et al. investigated the potential of a more homogenous cell type, like hepatocytes, to differentiate into functional neurons by using a specific combination of transcription factors (Marro et al., 2011).

All the above findings have major implications for cell-replacement strategies in various diseases, disease modeling and developmental studies.

1.8 Differentiation towards vascular lineages is governed by growth factors and the extracellular matrix

As it was previously mentioned, pluripotent cells can differentiate into vascular lineages responding to the right stimulus which can be cytokines or growth factors, the extracellular matrix, mechanical forces and communication with adjacent cells (Xiao et al., 2010). Previous studies in our laboratory have shown that ESCs can successfully differentiate into functional SMCs under an established differentiation protocol utilizing Collagen IV and platelet derived growth factor (PDGF) (Xiao et al., 2007). Hence, this section will focus on the role of PDGF and Collagen IV in the differentiation towards vascular lineages.

1.8.1 PDGF and its role in SMC differentiation

Cytokines are molecules released from progenitor/stem cells or mature SMCs and in turn stimulate other stem cells or progenitor cells to differentiate into specific lineages. Recent studies have indicated that among others, vascular endothelial growth factor (VEGF) and PDGF-BB are two factors essential for the development and maturation of the vascular lineages. which is produced close to forming vessels (Rafii, 2000). VEGF is a key molecule in vascular development which acts as a survival factor and a mitogen for vascular ECs (Isner, 2001). Administration of the above factors has lead to differentiation of stem cells into vascular cells. VEGF induced expression of EC markers such as von Willebrand factor (vWF) while the addition of PDGF-BB increased the expression of SMC markers such as smooth muscle actin (SMA), Calponin and smooth muscle myosin heavy chain (SMMHC) (Gerecht-Nir and Itskovitz-Eldor, 2004). Platelet-derived growth factor (PDGF) is a potent mitogenic and

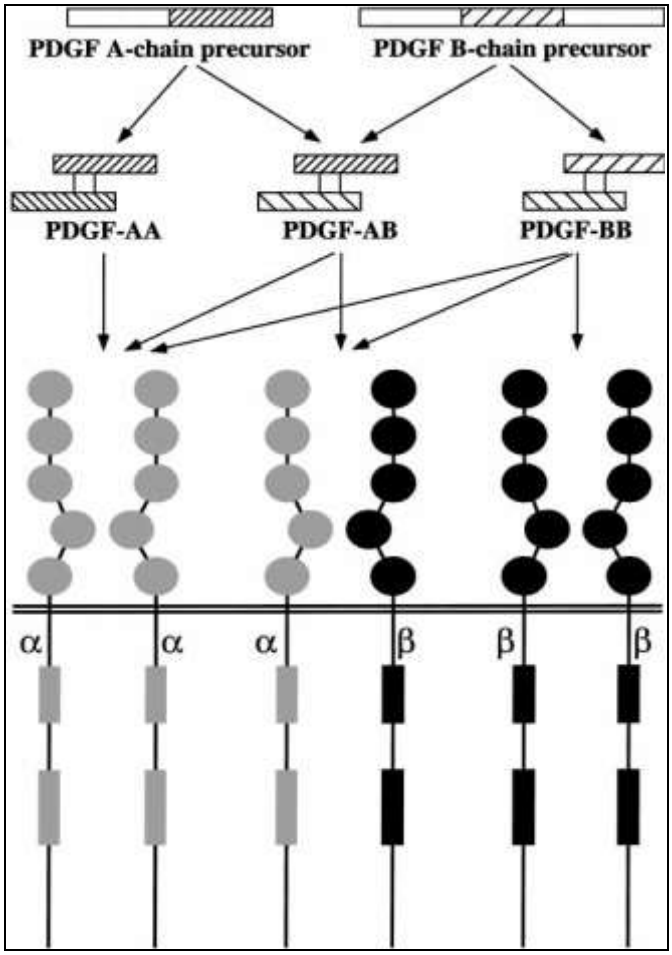
migratory factor that regulates the tyrosine phosphorylation of a variety of signalling proteins via intracellular production of H_2O_2.

It is a major mitogen for fibroblasts, SMCs and other cell types. Although a-granules of platelets are a major storage site for PDGF, further studies have shown that it can be synthesized by a number of different cells. It is a dimeric molecule consisting of 2 structurally similar A and B polypeptide chains which can combine into homo- or heterodimers (PDGF-AA, PDGF-BB, PDGF-AB). These isoforms act by binding to and activating two structurally related protein tyrosine kinase receptors, receptor –α and receptor –β (Heldin and Westermark, 1999) (Figure 8).

Although PDGF is expressed at low levels in arteries from healthy adults, its expression is increased in conjunction with the inflammatory response that characterizes atherosclerosis. Studies in naturally occurring atherosclerosis and experimentally induced atherosclerosis revealed that the expression of PDGF and PDGF receptors appears to be increased in these lesions. These observations suggest that PDGF, produced by activated macrophages, SMCs or ECs or released from platelets in thrombi, is important for the lesion formation (Heldin and Westermark, 1999).

Members of the PDGF family have been implicated in different pathophysiological conditions such as vascular development, neointima formation, wound healing during atherogenesis and atherosclerosis. For instance, inhibition of PDGF or its receptors in adults have reduced neointimal hyperplacia in response to injury (Raines, 2004). In addition to the above roles of PDGFs, PDGF-BB has been associated with induction of SMC and mural cell differentiation from bone marrow cells (Miyata et al., 2005).

However, its role in mature SMCs appears to be completely different since it negatively regulates the transcription of multiple SMC differentiation marker genes, a clear indication of its involvement in the SMC phenotypic switching (Dandre and Owens, 2004).



(Heldin and Westermark, 1999)

Figure 8. Processing and action of platelet-derived growth factor (PDGF) isoforms. A- and B-chains of PDGF are synthesized as precursor molecules that form disulfide-bonded dimers and undergo proteolytic processing. Different PDGF isoforms bind to and dimerize a- and b- receptors with different specificities. Intracellular parts of receptors contain tyrosine kinase domains, with characteristic inserted sequences without homology to kinases.

1.8.2 Collagen IV

The extracellular matrix (ECM) seems to be as critical in directing differentiation of pluripotent cells as cytokines and other soluble factors. The ECM is a complex structural entity which surrounds and supports cells that are found within mammalian tissues. It is an important factor affecting a variety of cell functions such as adherence, growth, migration, apoptosis and differentiation (Choy et al., 2004; Flaim et al., 2005; Hutchings et al., 2003). Collagens are the most abundant proteins in the animal kingdom and the major components of ECM. There are at least 12 types of collagens, with Collagen IV appearing important for vascular biology.

Collagen IV forms a two-dimensional reticulum and is a major component of the basal lamina (a layer secreted by the epithelium upon which the epithelium resides). In normal embryogenesis, ESCs differentiate along different lineages in the context of complex three dimensional (3D) tissue structures. It has been demonstrated that a 3D collagen matrix can mimic the aforementioned 3D tissue structure *in vitro*, inducing differentiation of rhesus monkey stem cells into various cell lineages (Chen et al., 2003). It has also been shown that Collagen IV has a crucial role in the early stages of differentiation of F9 stem cells (Watanabe et al., 2002). Finally, additional studies have within our laboratory have shown that cells positive for the VEGF receptor ($VEGFR^{+}$) can differentiate into SMCs when cultured on a Collagen IV substrate in α-minimal essential medium (α-MEM) containing 10% FBS. It was demonstrated that Collagen IV has a functional role in SMC differentiation of ES cells and that the Collagen IV-integrin signalling pathways mediate SMC differentiation from Sca-1+ progenitor cells (Xiao et al., 2007).

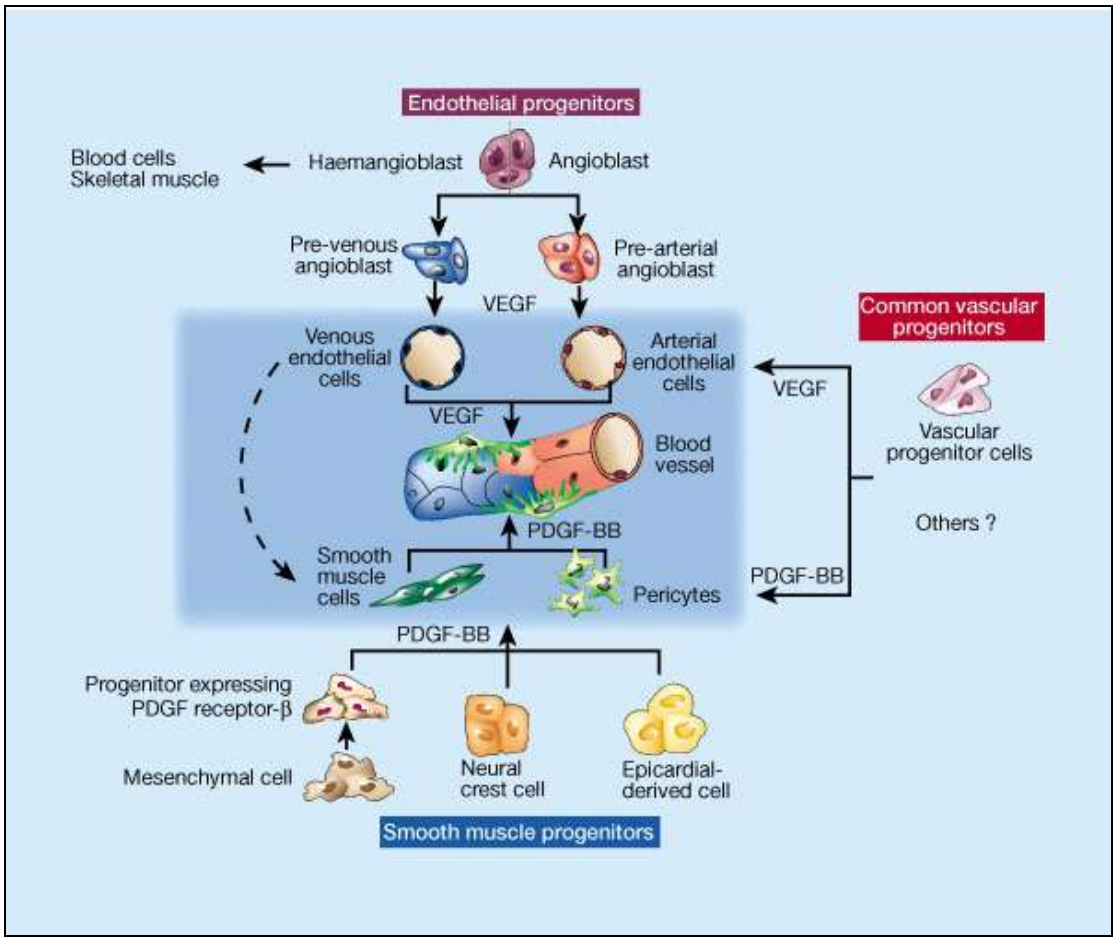
1.9 The vascular system

The cardiovascular system is one of the largest organs in the body, involved in the transport of blood and nutrients to the various tissues of the body as well as the removal of waste products and carbon dioxide. It is also the first organ to be formed in the developing embryo since, as soon as an embryo grows bigger than about 2 mm, it critically depends on a functional vasculature because passive diffusion is not sufficient to supply all cells with oxygen and nutrients. Blood cells and vessels arise from common precursor cells (hemangioblasts) which differentiate into blood cell precursors and vascular precursors (angioblasts). These angioblasts then migrate, ultimately forming a lumen. This process of vessel formation is called vasculogenesis and is dominant in very early embryogenesis (Risau and Flamme, 1995). The generation of additional vessels sprouting from pre existing vessels is called angiogenesis. It can be induced by hypoxia and can also occur in multiple diseases including many forms of cancer (Carmeliet, 2003) as well as arteriosclerosis, which involves remodelling of the existing vasculature and expansion of collateral vessels (Carmeliet, 2000b).

Blood vessels are generally composed of two cell types: ECs which form the intimal region of the vessel and SMCs which surround the intima, generating the medial layer and controlling the blood flow. Larger blood vessels also contain a third layer of cells (fibroblasts) which form the adventitia (Figure 10). However, vessels within the microvascular system only contain an intimal region and cells not joined in layers, called pericytes, which cover a sporadic region of the endothelial cells (Carmeliet, 2000a).

Initially, it was considered that endothelial and SMCs arise from separate 'precursor' cells through rounds of cell division and specialization, however, in 2000 Yamashita and his colleagues described a type of blood-vessel precursor from which both ECs and SMCs could be developed *in vitro* and *in vivo* (Yamashita et al., 2000)

Endothelial precursors have also been found in adults and most of them divide and differentiate in response to VEGF (Figure 9).



(Carmeliet, 2000a)

Figure 9: A common origin for the two types of blood-vessel cells. Centre, ECs are the cells that make up the interior walls of a blood vessel. SMCs or pericytes encase the endothelial channels. These basic cell types have been thought to arise from separate types of precursor. ECs arise from precursors called angioblasts or haemangioblasts in the embryo (top), or from circulating endothelial progenitors in the adult (not shown). SMCs and pericytes, by contrast, can form from a variety of progenitors (bottom).

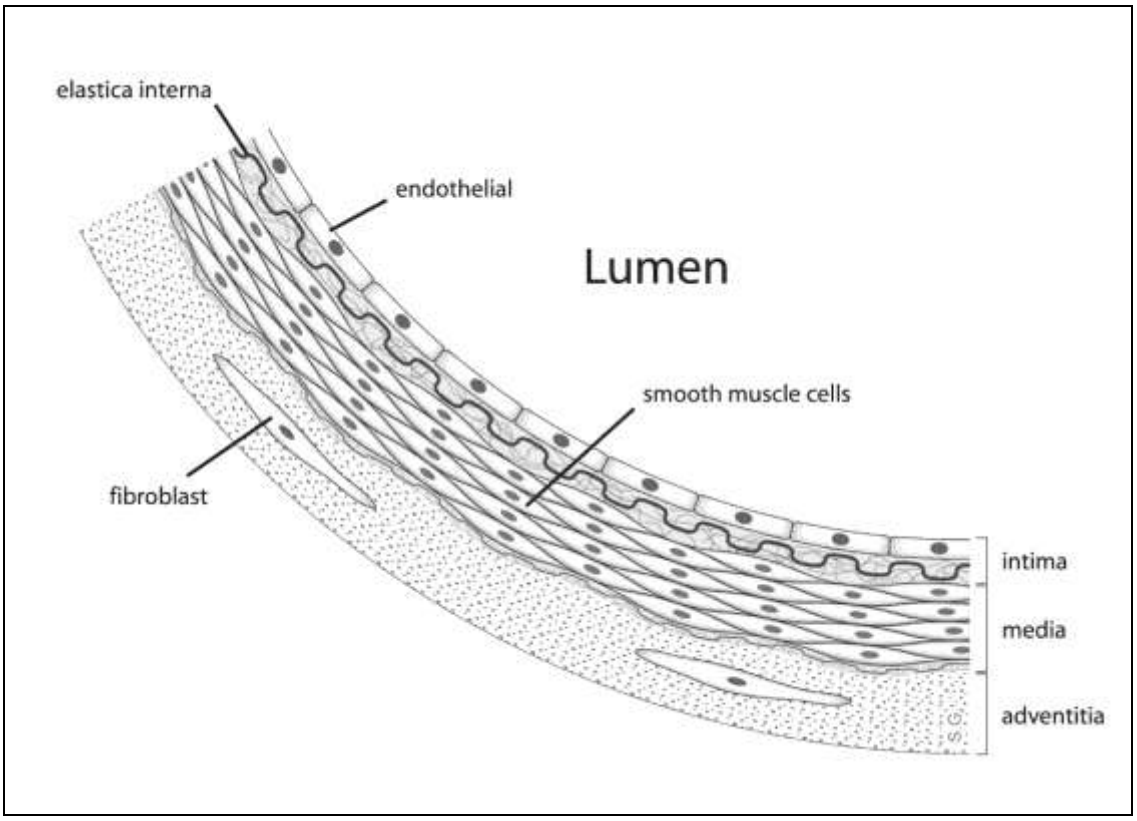


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Figure 10. Anatomy of the arterial wall. Schematic representation of the arterial wall and the layers it is composed of. The endothelial cells comprise of the intima, the inner most layer of the vessel while the contracting SMCs are part of the media layer and surround the ECs. Bigger vessels also contain an additional layer containing fibroblasts (adventitia).

1.10 Vascular SMCs

Vascular SMCs have a critical function in maintaining the integrity of the vascular system. Heterogeneity both during development as well as in the adult vasculature is one of the most characteristic features of vascular SMC. However, when talking about heterogeneity the basic question of whether we are dealing with a SMC heterogeneity in origin or with a spatiotemporal heterogeneity in expression of differentiation markers should be addressed (Majesky, 2007).

Lineage tracking studies have revealed that vascular SMCs in different vessels have distinct embryological origins (Majesky, 2007). In the developing embryo, the first mural cells investing the endothelial tubes derive from transdifferentiated endothelium (mesodermal origin) during nascent vascular and cardiac valve development (Nakajima et al., 1997). Subsequently, there is evidence of additional sources of origin of SMCs. For example, vascular SMCs in coronary arteries derive from the epicardial lining in a similar way to the transdifferentiation of adventitial cells of the coronary vessel wall. However, SMCs from the aortic arch are likely of neuroectodermal origin namely, from the mesectoderm of the neural crest and the cells constituting the descending aorta originate predominantly from the local mesenchyme (Gittenberger-de Groot et al., 1999). The diversity of SMC origins may in part contribute to the site specific localization of vascular diseases and explain the regional susceptibility to atherosclerosis (DeBakey et al., 1985; Haimovici and Maier, 1964)

During the late fetal and post natal development, SMCs are characterized by a very high proliferative rate and by the expression of significant and unique serum-independent growth capabilities *in vitro* (Cook et al., 1994).

Furthermore, there is a rapid induction in the expression of multiple mature SMC marker genes as well as high rates of synthesis of ECM components such as collagen, elastin, proteoglycans, cadherins and integrins, which comprise a major portion of the blood vessel mass. Therefore, this stage of development (synthetic) is crucial for the formation of abundant gap junctions with ECs as well as the process of investment of endothelial tubes with SMCs or pericytes, an important process towards vessel maturation and remodelling (Hungerford and Little, 1999).

In contrast to the embryonic and fetal/postnatal phenotypes, in the adult blood vessels SMCs display an exceedingly low proliferative rate, a mainly non-migratory phenotype and an extremely low rate of synthesis of ECM components. They have now become highly specialised cells responsible for contracting and regulating the blood tone-diameter, blood pressure and blood flow distribution with higher densities in arteries when compared to veins (Owens, 1995). In this fully differentiated state, they express a subset of appropriate receptors, ion channels, signal transduction molecules, calcium regulatory proteins and contractile proteins necessary for the unique contractile properties of the SMC (Owens et al., 2004).

Upon vascular injury the contractile “non synthetic” SMCs can undergo a phenotypic switch as a response to appropriate cues and transform into the synthetic phenotype, playing a critical role in the repair of the vascular injury. After successful repair and again in response to the appropriate cues, they will re-aquire their contractile phenotype and return to normal. (Owens et al., 2004). The factors that regulate the expression and loss of this SMC growth phenotype are likely to be important components of a developmental system controling vascular morphogenesis and possibly SMC replication is vascular disease states (Cook et al., 1994).

Despite compelling evidence that phenotypic modulation of the SMC plays a key role in vascular injury repair and in the development and/or progression of atherosclerosis, relatively little is known about how this process is regulated *in vivo*.

Although it is now known that SMC derive from at least 8 independent vascular SMC progenitors, it is not yet clear how they can later become specified for a common smooth muscle fate and how they are maintained as smooth muscle myoblasts during the migration, proliferation and heterotypic cell-cell interactions required to position them for differentiation around nascent blood vessels (Majesky, 2007). Moreover, recent evidence suggests that in perinatal and adult vessels, SMC progenitor cells reside in a privileged signalling domain or niche environment within the tunica adventitia (Hu et al., 2004). Their persistence in this perivascular location throughout adult life suggests that cells and matrix components of the adventitial niche provide important signals that maintain the progenitor phenotype and prevent premature SMC differentiation (Passman et al., 2008).

It is now known that maintenance of SMC progenitor pools requires signals that allow for proliferative expansion during tissue development, growth and repair together with activation of redundant transcriptional silencing mechanisms to maintain the SMC progenitor phenotype and prevent SMC differentiation. However, a more complete understanding of the molecular mechanisms used to form and maintain vascular SMC progenitor cells is both fundamentally important at a basic level and will enable advances such as the iPS cell technology to be more effectively applied to disorders of the vessel wall (Majesky et al., 2011).

1.11 Neovessel formation

The process of neovessel formation is an important event both during embryonic development as well as in the adult tissues where ischemic infarction occurs. Neovessels from the neighbouring normal tissues are needed to form the vessel network and restore the blood supply to the damaged tissues. Both ECs and SMCs are essential. However, the detailed mechanism of SMC migration and differentiation is not fully understood.

Until recently, it was accepted that vessels in adult ischemic tissues could only grow by angiogenic mechanisms, i.e. the sprouting of mature ECs from pre-existing vessels, likely in response to angiogenic factors. However, recent studies have revealed that endothelial progenitor cells (EPCs) circulate postnatally in peripheral blood. These may be recruited from the bone marrow and incorporate into sites of active neovascularisation in ischemic hindlimbs, ischemic myocardium, injured corneas and tumor vasculature (Asahara et al., 1999). This process is termed postnatal vasculogenesis (Luttun et al., 2002).

It has previously been indicated that adult bone-marrow-derived mesenchymal stem cells (BMSCs/MSCs) and multipotent adult progenitor cells (MAPCs) can be differentiated into endothelial-like cells *in vitro* and contribute to neoangiogenesis *in vivo* (Al-Khaldi et al., 2003; Oswald et al., 2004; Reyes et al., 2002). Furthermore, BMSCs can increase collateral remodelling and perfusion in ischemic models through paracrine mechanisms rather than by cellular incorporation upon local delivery (Kinnaird et al., 2004).

Recently, it has been shown that adult BMSCs, under appropriate *in vitro* environmental cues, can be induced to undergo vasculogenic differentiation culminating in microvessel morphogenesis. When rat BMSCs were seeded onto a three-dimensional (3D) tubular scaffold, the maturation and co-differentiation into endothelial and/or SMC lineages which lead to successful microvessel formation was observed (Valarmathi et al., 2009).

A separate study showed that locally delivered, activated cardiac progenitor cells (CPCs), could generate *de novo* coronary vasculature by dividing and differentiating in both ECs and SMCs, restoring blood supply to ischemic myocardium (Tillmanns et al., 2008).

Elucidation of the contribution of each vascular population to neovascularisation as well as the cues which stimulate their maturation or/and differentiation is essential for their future therapeutic prospective.

1.12 Tissue engineering of vascular grafts

Cardiovascular disease is the leading cause of death in many developing countries. Each year, more than 570.000 coronary artery bypass graft procedures are performed in the Unites States alone, creating an important demand for small diameter (<6 mm) vascular grafts (Mitchell and Niklason, 2003). The challenge of tissue engineering blood vessels with the mechanical properties of native vessels, and with the anti-thrombotic properties required, is immense. Recent advances, however, indicate that the goal of providing a tissue-engineered vascular graft that will remain patent *in vivo* for substantial periods of time, is achievable (Ratcliffe, 2000).

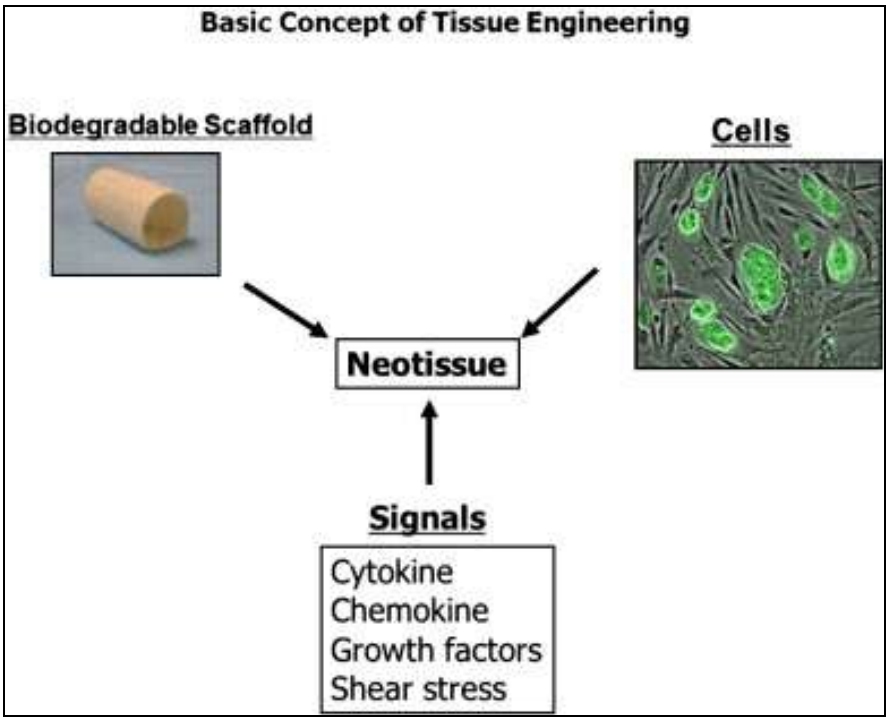
The basic concept of tissue engineering consists of three basic components, which include: a) cells (either seeded *in vitro* or mobilized *in vivo*), b) scaffolds onto which the extracellular matrix is organized in neotissue formation and c) signals (humoral and mechanical) (Bell, 1991). All three factors are interdependent and are indispensable to the formation of highly organized vascular tissue.

The synthetic vascular grafts currently used have several limitations including thrombogenicity, increased risk of infection, and lack of growth potential. However, the first clinical trial evaluating the feasibility of using tissue engineered vascular grafts (TEVG) created by seeding autologous bone marrow-derived mononuclear cells (BM-MNC) onto biodegradable tubular scaffolds has recently been completed. Naito *et al* showed that despite an excellent safety profile the primary graft related complication affecting approximately 16% of grafts within the first seven years after implantation of the TEVG, is stenosis, (Naito et al., 2011).

In a different study, banked porcine SMCs that were allogeneic to the intended recipient were used to generate tissue engineered vessels (TEVs) by utilization of a biomimetic perfusion system. The vessels were decellularised, leaving behind the mechanically robust extracellular matrix of the graft wall and then seeded with cells that were derived from the intended recipient (either EPCs or ECs) on the graft lumen. TEVs were then implanted in the porcine carotid artery. The results revealed that the generated graft is capable of resisting both clotting and intimal hyperplasia and that engineered connective tissues can be grown from banked cells, rendered acellular, and then used for tissue regeneration *in vivo* (Quint et al., 2011)

Finally, in one of the most recent studies, sheets created from iPS cell derived vascular cells were evaluated as potential sources for the construction of TEVGs. It was shown that differentiated iPS cells provide an alternative and attractive cell source for constructing TEVGs using the sheet engineering technique and that sheet seeding of TEVG represents a viable model of iPS cell delivery over time (Hibino et al., 2012).

The development of the TEVG represents a major breakthrough for the future of medicine. Although basic science innovations and clinical research studies will be needed for continued improvement of this exciting area, the combination of tissue engineering and iPS cell technology holds great promise in the future of regenerative medicine.



(Naito et al., 2011)

Figure 11. The basic concept of tissue engineering. The three components required for the construction of a TEVG are cells, scaffolds onto which the extracellular matrix will be organized to support the neotissue and finally the appropriate signals which are indispensable for the formation of an organized tissue.

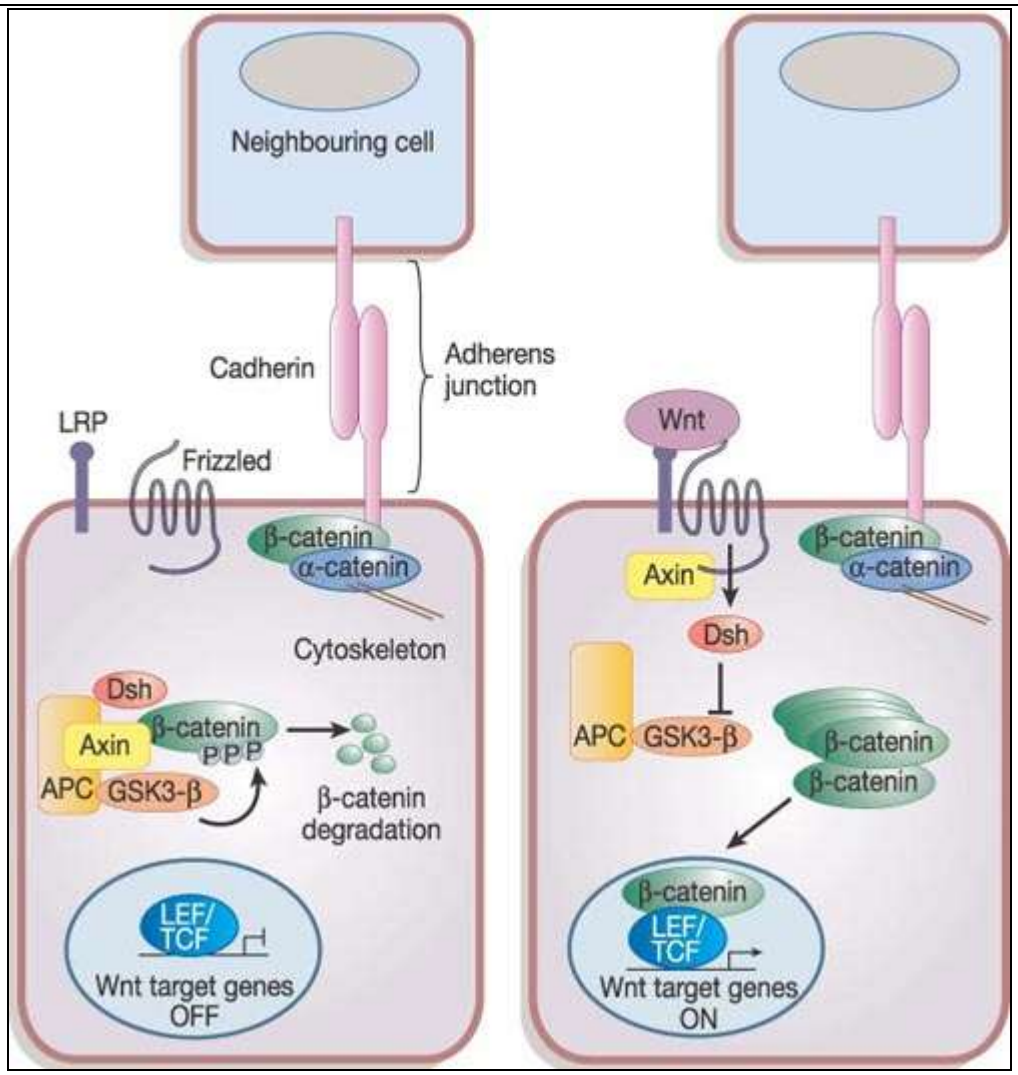
1.13 Wnt signalling in the vascular system

Wnt signalling is a key regulator of multiple aspects of tissue development. It comprises of a group of cysteine-rich glycoproteins which regulate cell proliferation, cell morphology, cell motility and cell fate (Dale, 1998).

An increasing body of evidence indicates that the Wnt signalling pathway is likely to play an important role in angiogenesis and vessel remodelling, with reports from a variety of investigators pointing to a role of Wnt signalling in the vasculature in both normal and pathologic vessel growth (Goodwin and D'Amore, 2002). It has recently been shown that activation of Wnt signalling in mouse $Flk1^+$ cells (cell with a developmental potential restricted to hematopoietic and endothelial lineages) was able to expand vascular progenitor populations, whereas suppression of Wnt activity reduced $Flk1^+$ populations and inhibited the formation of matured vascular capillary-like structures during late stages of embryoid body differentiation (Wang et al., 2006). Furthermore, recent studies have demonstrated that the ligand Wnt7b regulates a program of mesenchymal differentiation through a Wnt/Tenacin C/ PDGFR signalling axis in the mouse lung that is essential for SMC development and disease progression in the lung (Cohen et al., 2009).

Although there are three branches to the Wnt signalling pathway, only the canonical Wnt signalling (or otherwise known as β catenin pathway) will be mentioned for the purposes of this thesis. A simple outline of the current model of the canonical Wnt signal transduction is presented in Figure 12.

In vertebrates, several secreted proteins have been described, that can modulate Wnt signalling by either binding to Wnts or binding to a Wnt receptor protein. The extracellular antagonists of the Wnt signalling pathway can be divided into two groups both of which prevent ligand-receptor interactions, but by different mechanisms. The members of the first class, which include the sFRP (secreted Frizzled-related protein) family, WIF (Wnt inhibitory factor)-1 and Cerberus, primarily bind to Wnt proteins. The second class comprises certain members of the Dickkopf (DKK) family, which bind to one subunit of the Wnt receptor complex. However, certain sFRPs and DKKs do not antagonise Wnt function (Kawano and Kypta, 2003).



(Reya and Clevers, 2005)

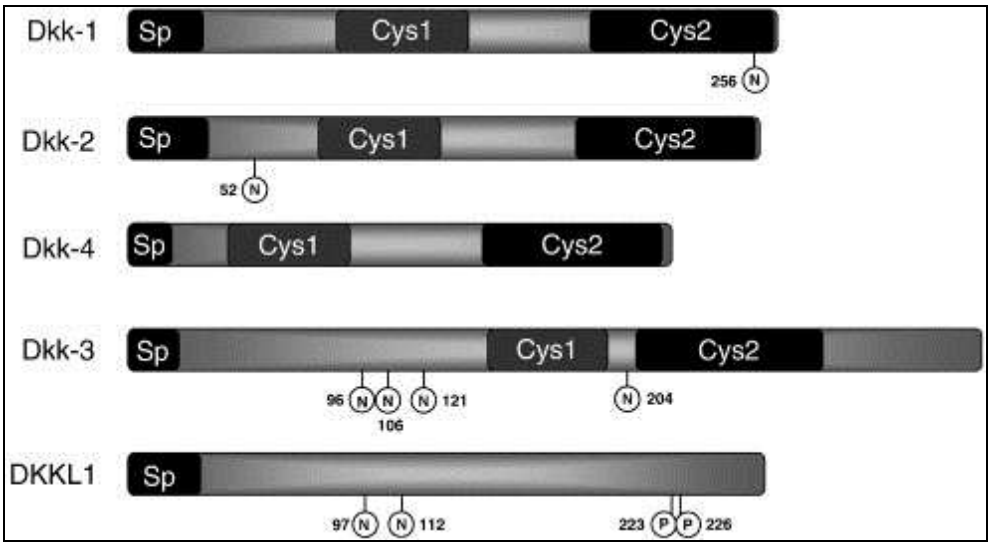
Figure 12. The canonical Wnt signalling pathway. In the absence of Wnt signalling (left panel), β catenin is in a complex with Axin, APC and GSK3-β, where it is subjected to phosphorylation and is targeted for degradation. β catenin also exists in a cadherin-bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), β catenin is uncoupled from the degradation complex and translocates to the nucleus, where it binds the Lef/Tcf transcription factors, thus activating target genes.

1.14 DKK3, a member of the Dickkopf family of Wnt inhibitors

1.14.1 The DKK family

The human DKK family comprises of four members, DKK1-4 and a unique DKK3-related protein SOGGY (DKKL1 or SGY1). DKK proteins are secreted glycoproteins with a molecular weight between 25kDa and 29kDa for DKK1, DKK2 and DKK4 or 38kDa for DKK3. All the members of the family share an N-terminal signalling peptide and two concerved cystein-rich domains (CRD) (Cys1 and Cys2) separated by a linker region, while other members of the family show only weak amino acid (aa) sequence similarities (Figure 13) (Glinka et al., 1998). The Cys1 domain is unique to the DKK family and is not found among other vertebrate proteins whereas the Cys2 domain pattern relates to a functional domain called colipase fold. Colipases are characteristic for lipid hydrolysis and for that reason it has been suggested that Cys2 in DKKs may enable proteins to interact with lipids and thus regulate Wnt function (Aravind and Koonin, 1998). Colipase folds can also be found in toxins and protease inhibitors (Kaser et al., 2003; Szeto et al., 2000). No functional domains have been identified in SGY and to date its biological role is mainly restricted to its expression in human spermatogenesis where it mediates pubertal spematocyte apoptosis (Kohn et al., 2005). Finally, in contrast to the highly conserved CRDs, the linker region between Cys1 and Cys2 is highly variable between DKKs and it is notably larger in DKK1, DKK2 and DKK4 (52-55aa) whereas it is only 12aa in DKK3 (Krupnik et al., 1999).

Furthermore, DKK3 contains four potential N-glycosylation sites which are not conserved in the other members and contains an extended N-terminal domain which precedes Cys1 and an extended C-terminal domain following Cys2 making DKK3 the most divergent member of the family (Krupnik et al., 1999).



(Veeck and Dahl, 2012)

Figure 13. Structural homology of the human DKK family. DKK proteins contain two highly conserved cysteine-rich domains (Cys1 and Cys2), which are separated by a non-conserved linker region of variable length. Within the DKK family, the structural diversity of DKK3 is pronounced by its larger protein size, a shorter linker region and a high N-terminal sequence similarity with DKKL1, but not with DKK1, DKK2 or DKK4. Additionally, DKK3 possesses four putative posttranslational N-glycosylation sites, whereas DKK1 and DKK2 only harbor one site. Numbers refer to the modified amino acid residue. In the C-terminal region of DKKL1 two serine residues are phosphorylated.

1.14.2 The biological role of DKK3

Developmental studies in mural embryos have demonstrated that expression of the DKK family members is temporally and spatially regulated with overlapping expression patterns in mesenchymal lineages, suggesting a coordinated mode of action and cell fate decisions in tissues mediating epithelial-to-mesencymal transition (Monaghan et al., 1999). Furthermore DKK3 seems to have a decisive function in myogenic cell fate since it was recently found to be highly expressed in different skeletal muscle subtypes. (de Wilde et al., 2010). Further evidence for a role in myogenesis adds from the evidence that DKK3 negatively regulates expression of myf5, a transcription factor regulating muscle differentiation. Interestingly, a regulatory microRNA (miR-ln300) encoded in the first intron of the myf5 gene directly downregulates DKK3, pointing to an interesting negative feedback loop between DKK3 expression and muscle tissue differentiation (Hsu et al., 2010).

Concerning Wnt signalling, while DKK1, DKK2 and DKK4 negatively regulate the aforementioned pathway by binding to LRP5/6 or the Kremen co-receptor proteins with high affinity, to date, no corresponding cell surface membranous receptor has been identified for DKK3 (Mao et al., 2002; Mao et al., 2001; Semenov et al., 2001). Although DKK3 inhibited Wnt7a in pheochromocytoma PC12 cells(Caricasole et al., 2003) and also exhibited a Wnt-inhibitory activity in the osteosarcoma Saos-2 cell line(Hoang et al., 2004), it was unable to inhibit Wnt reporter Tcf-luciferase acrivity in the prostate cancer cell line LNCaP(Kawano et al., 2006).

Furthermore, it was shown that ectopic expression of DKK3 in prostate cancer cells, lead to apoptosis via c-Jun NH_2-terminal kinase (JNK) activation suggesting a role of DKK3 in the Wnt/PCP (polar Wnt pathway) and not in the canonical Wnt signalling pathway (Abarzua et al., 2005). In addition to its secretory role, DKK3 could also have an intracellular function, since it was recently shown that there is a direct interaction between DKK3 and βTrCP (a negative regulator of β catenin). In this study, a new model of cytosolic Wnt inhibition where DKK3 directly targets β catenin was suggested (Lee et al., 2009). Finally, it was recently shown that DKK3 can potentiate Wnt3a through interactions with the transmembrane protein Kremen 1, a protein located in intracellular membranus compartments such as the endoplasmatic reticulum (ER) (Nakamura and Hackam, 2010).

Therefore, it can be concluded that the ability of DKK3 to inhibit or potentiate Wnt signalling as well as its effect on a particular Wnt pathway branch appears to be context-specific. Further studies are necessary in order to elucidate the precise mechanism by which DKK3 interferes with Wnt signalling.

1.14.3 DKK3 and its role in cancer

DKK3 or otherwise known as REIC (Reduced Expression in Immortalised Cells) was first implicated with tumorigenesis when during the search for immortalization-related genes, Tsuji and his colleagues found a gene that was downregulated in immortalised cell lines and a number of established human cancer lines. Cloning of the cDNA and analysis of the predicted amino acid sequence revealed that that gene was identical to the human DKK3 (Tsuji et al., 2000).

Soon thereafter, the differential expression of DKK3 was confirmed in lung cancer where in 57 matched pairs of NSCLC (non-small cell lung cancer) and adjacent normal lung tissues, it was revealed that DKK3 mRNA expression was substantially reduced in 63% of the tumor specimens (Nozaki et al., 2001). This was followed by identification of DKK3 downregulation in virtually any human solid cancer type including prostate, colon and breast cancer (Abarzua et al., 2005; Veeck et al., 2008; Yu et al., 2009). Although other member of the DKK family are also differentially expressed in certain tumor tissues, they are not consistently downregulated like DKK3 which emphasizes the exceptional role of DKK3 within the family (Veeck and Dahl, 2012).

The molecular mechanisms by which DKK3 expression is abolished in human cancers has been comprehensively studied. The first report about the DKK3 gene regulation showed that its promoter is hypermethylated in lung cancer cell lines as well as in a large fraction of the NSCLC tissues with significant loss of DKK3 expression in these samples (Kobayashi et al., 2002).

DKK3 methylation-mediated suppression was assessed in numerous tumor entities where it was revealed that DKK3 methylation is a tumor-specific event which is absent or significantly lower in non-malignant tissue (Chim et al., 2007; Ding et al., 2009; Fujikane et al., 2010; Lodygin et al., 2005; Veeck et al., 2008; Yang et al., 2010; Yu et al., 2009; Yue et al., 2008). Additionally, global DNA epigenetic demethylation by epigenetic drugs revealed that DNA methylation is directly associated with gene expression loss of DKK3, where it was shown that abolished DKK3 expression was reversible in a number of cancers (lung/breast/gastric/prostate cancer and leukemia) except from malignant melanoma cells (Kobayashi et al., 2002; Kuphal et al., 2006; Lodygin et al., 2005; Valencia et al., 2009; Veeck et al., 2008; Yu et al., 2009). Furthermore, it has recently been reported that post-transcriptional factors may play a role in suppression of DKK3. Two independent studies revealed that upregulation of the oncogene MYCN lead to the upregulation of microRNA clusters which directly targeted the 3'UTR sequence of the DKK3 mRNA and inhibited its translation in neuroblastoma cells (De Brouwer et al., 2011; Haug et al., 2011)

Finally, there is compelling *in vitro* and *in vivo* evidence that DKK3 is a natural tumor suppressor gene in human tissues. The most evident and consistent anti-tumor effect of DKK3 is that of its inhibitory capacity on cancer cell growth, as determined by proliferation and anchorage-independent growth assays in a variety of cancer cell line models (Veeck and Dahl, 2012). The consistent loss of expression of DKK3 in the various tumor entities as well as its ability to strongly inhibit tumor growth, qualify DKK3 as a potential therapeutic target in cancer disease.

1.14.4 The role of DKK3 in tumor angiogenesis

In order to meet the elevated need for oxygen and nutrients, growing tumors require an extensive vasculature. Therefore they stimulate surrounding stromal tissue to produce novel blood vessels, a process which is known as tumor angiogenesis (Folkman, 1971). RNA expression analysis of normal and tumor activated endothelium revealed that DKK3 was one of the most strongly upregulated genes in the tumor endothelium of colorectal cancer (Untergasser et al., 2008). DKK3 exerted a differentiating effect on tumor-associated ECs while still clearly acting as a growth inhibitor on epithelium-originated tumor cells. Therefore, it was considered a putative pro-angiogenic factor in vascularization and a possible marker for neo-angiogenesis (Zitt et al., 2008). Further studies revealed that in comparison to normal tissue, there was a higher number of DKK3 positive vessels in a variety of cancers (Veeck and Dahl, 2012). Finally, *in vitro* cell proliferation and migration assays revealed that while endothelial colony forming cells (ECFCs) were not affected by DKK3 expression, tube formation in Matrigel® increased after enforced DKK3 expression and deceased after its downregulation. Additionally, stable overexpression of DKK3 in B16F10 melanoma cells significantly increased microvessel density in the C57/BL6 melanoma mouse model suggesting that DKK3, suggesting the involvement of DKK3 in the remodelling of the tumor vasculature, as a differentiation factor (Untergasser et al., 2008). Taking the above studies under consideration, it would therefore be interesting to further examine the role of DKK3 in vascular associated processes and particularly in vascular differentiation.

1.15. Hypothesis and aim of the study

The generation of iPS cells is a gradual process where terminally differentiated cells are transformed into pluripotent cells capable of recapitulating the characteristics of ESCs. The utilisation of a cocktail of transcription factors as well as the epigenetic modifications that occur in the somatic cells during that transition, suggested that a cascade of events such as activation or de-activation of signalling pathways takes place in order to eventually lead the cells to pluripotency. The inception of this project originated by the hypothesis that within the aforementioned cascade of events, specific pathways could be activated early during the reprogramming, which would lead to direct differentiation towards specific lineages, in particular SMCs, prior to the cells reaching the pluripotent stage. That would mean that given the appropriate stimulus after an initial "reprogramming activation" phase, somatic cells would be directly differentiated into SMCs by bypassing the pluripotency stage.

In order to test that hypothesis, a microarray analysis of cells in different time points during the reprogramming was conducted, where it was identified that the expression of a great number of genes associated with key functions of the cell, was altered from as early as day 4. Therefore, the aim of the present study was to initially investigate **the ability of 4 days reprogrammed cells to differentiate into SMCs by bypassing pluripotency.** Specifically, the 4 days reprogrammed cells were characterised and named Partially induced Pluripotent Stem cells (PiPS). They were then placed under established SMC differentiation conditions as previously shown in our laboratory for differentiation of ESCs to SMCs (Xiao et al., 2007).

The differentiation potential of PiPS to SMCs was therefore investigated. A schematic representation of the experimental design is presented in Figure 14.

Successful differentiation of PiPS cells into SMCs lead to investigation of the molecular mechanisms which regulate the induction of SMC markers. DKK3, a gene indicated by the microarray to have an altered expression during the reprogramming and also previously shown to be associated with neo-angiogenesis in cancer vasculature (Zitt et al., 2008) was found to be significantly upregulated during the PiPS-SMC differentiation. Therefore, in this part of the study the **role of DKK3 in PiPS-SMCs differentiation as well as the signalling pathway by which DKK3 is regulating SMC gene expression** was investigated. The experimental design included overexpression, stimulation and silencing of DKK3 during PiPS-SMCs differentiation, investigation of its effect on the Wnt signalling pathway with which it has previously been associated (Mao et al., 2002) as well as examination of the transcriptional regulation of the SMC specific marker SM22.

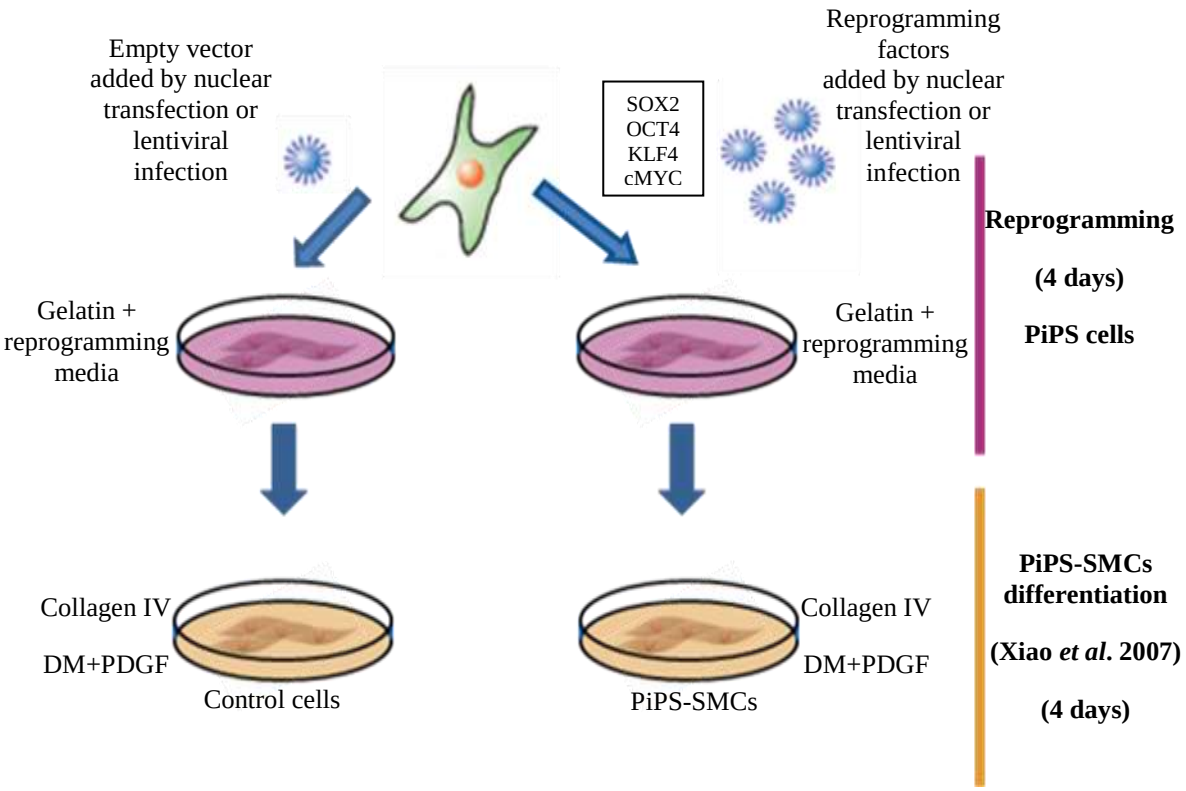


Figure 14. Schematic representation of the protocol used to generate PiPS-SMCs. Human neonatal fibroblasts were forced to overexpress the four transcription factors and maintained in reprogramming media for 4 days on a gelatine substrate. An empty vector is used as a mock control. After 4 days of reprogramming (PiPS cell stage) the cells are transferred on a Collagen IV substrate and maintained in DM containing PDGF where they are differentiated for 4 days and subsequently harvested for further analysis.

Chapter 2
Materials and Methods

2.1 Materials

Cell culture media, serum and cell culture supplements were purchased from ATCC, Invitrogen, Life technologies and PAA. Basic FGF-2 was purchased from MACS (Miltenyi Biotech Cat No: 130-093-837), aliquoted into 10.000x stock solutions of 100µg/mL in sterile water and stored at -20^{o}C. PDGF was purchased from R&D systems (Cat No: 220-BB), aliquoted into 10.000x stock solutions of 100µg/mL in sterile 4mM HCL and stored at -20^{o}C. Human recombinant DKK3 cytokine was purchased from R&D systems (Cat No: 1118-DK), aliquoted into 5000x stock solutions of 250µg/mL in sterile PBS and stored at -20^{o}C. Purchased antibodies used for immunoblotting and their recommended dilutions are shown in Table 5. Primary antibodies used for immunoblotting were diluted in PBS containing 5% milk, 0.05 Tween® 20 (Sigma-Aldrich, Cat No P2287) and 0.02% sodium azide and stored at -20^{o}C. Buffers made for the methods herein are shown in Table 6.

2.2 Methods

2.2.1 Cell culture of human embryonic fibroblasts

Prenatal human embryonic fibroblasts purchased from ATCC (catalogue number: CCL-153™) were cultured on gelatin coated flasks (0.04% of 2% Solution Type B from Bovine Skin, Sigma G1393) in ATCC F-12K Medium (Kaighn's Modification of Ham's F-12 Medium, Cat No: 30-2004) supplemented with 10% ATCC Fetal Bovine Serum (Cat No: 30-2020) and 100 U/mL penicillin and streptomycin in a humidified incubator supplemented with 5% CO_2. Cells were passaged every three days in a ratio of 1:3 or 1:7 and the medium was refreshed every two days.

2.2.2 Restriction digestions

A polycistronic plasmid (OSKM) containing all four factors (OCT4, SOX2, KLF4 and cMYC) on one vector, was purchased from Addgene (Plasmid 20866: pCAG2LMKOSimO (Kaji et al., 2009)) (Figure 15) and was used for the generation of Partially iPS (PiPS) as described in the following paragraph. Before transfection the plasmid was linearised with the PVU1 restriction enzyme for 2 hours at 37^{o}C and then purified using SureClean (Bioline, Cat No: 37042).

A restriction digestion was performed to generate a control vector from the aforementioned OSKM plasmid. Restriction enzymes BamHI and BspEI were used to remove the ORF from the pCAG2L backbone vector. The plasmid and enzymes were incubated at 37 ^{o}C for 2 hours. The samples were then loaded on a 1.5% agarose gel, electrophorated, and subsequently cloned (paragraph 2.2.4).

A restriction digestion was also performed to generate a control vector from the lentiviral TetO-FUW-OSKM (Figure 17). ClaI was utilised to remove the ORF from the Teto-FUW vector backbone. After incubation of the plasmid and enzymes at 37^{o} for 1 hour, the samples were electrophorated and subsequently cloned (2.2.4). The plasmid was purchased from Addgene Cat No 20321.

2.2.3 Agarose gel electrophoresis and gel purification

Agarose gels (1.5%) were prepared by melting 1.5g agarose (Invitrogen, Cat. No.15510027) for 2 minutes in an 800W microwave with 100ml 1x Tris-base Acetic acid EDTA (TAE) buffer diluted from a 50xTAE (National Diagnostics, Cat. No, EC-

872) stock (2.0M Tris Acetate plus 100mM Na_2 EDTA) conical flask.7.5µl of EtBr 10mg/ml (Sigma, Cat. No. E1510) were added to the solution to a final concentration of 0.75 µg/ml. The mix was then poured into the Horizon® 11.14 horizontal gel cast electrophoresis apparatus (Biometra) and was allowed to cool at room temperature for 30 minutes, after which it was immersed in 1xTAE. 5µl of 6x Loading Buffer (0.25% (w/v) Bromophenol blue, 0.30% (v/v) glycerol) was added per 25µl of the digested vector and then loaded into the wells.

The electrophoresis was performed at 160V for ~25 minutes visualised under ultraviolet light with the Biospectrum® Imaging System 500 and captured with the Vision Works Software (Ultra-Violet Products Ltd). The target fragments were identified and then cut and purified using the QIAgen Gel Extraction kit as instructed by the manufacturer (Qiagen Cat. No. 28704).

2.2.4 Cloning of pCAG2L-ctl and Teto-FUW-ctl (Ligation and transformation)

The digested vectors were self ligated in the presence of T4 DNA ligase (Promega, Cat. No. M18041) after overnight incubation at room temperature. Transformation was performed in JM109 competent cells (E.Coli) (Promega, Cat. No. L2001). The ligation reactions were incubated on ice and the competent cells were thawed for a few seconds. 100µl of competent cells were added to each ligation reaction and then incubated on ice for 30 minutes. Heat shock was then performed at 42 ^{o}C for 90 seconds after which the tubes were returned on ice. 1 ml of sterile L.B (Luria-Bertani) medium was added to each tube and incubated for 1 hour at 37 ^{o}C after which the tubes were centrifuged at 4000 rpm for 5 minutes.

850μl of the supernatant were discarded, the pellet was resuspended in the remaining L.B medium and then transferred on Ampicilin LB Agar sterile plates (50μg/ml) for overnight incubation at 37^{o}C. Colonies were picked up and amplified in LB Ampicilin medium (100μg/ml) overnight after which plasmid purification was performed using a QIAprep Spin Miniprep kit (Qiagen, Cat. No. 27016) as instructed by the manufacturer. Verification of the plasmids was performed by restriction digestion reaction after which DNA sequencing was employed for further verification.

2.2.5 Nucleofection of fibroblasts using the polycistronic OSKM plasmid or four expression vectors each carrying one transcription factor

The polycistronic OSKM plasmid was overexpressed in embryonic fibroblasts as part of the reprogramming method (Figure 15). Fibroblasts were transfected with the OSKM plasmid by electroporation using an NHDF nucleofector Kit (Lonza,Cat No: VPD-1001). The control (ctl) vector generated as described in paragraphs 2.2.2-2.2.4 was used to transfect fibroblasts using the same method. Each transfection required 2x10^{6} cells and 4μg of ctl or OSKM plasmid. Cells were washed with PBS, trypsinized and counted. Aliquots of 2x10^{6} cells per falcon tube were pelleted at 1000 rpm for 5min and the supernatant medium was discarded. The cell pellet was then resuspended in 100 μl of nucleofection solution containing the plasmid and the mix was transferred in a nucleofection cuvette as provided by the company. The cuvette was then placed in the Lonza nucleofection machine and a specific for NHDFs nucleofection program was used. 500μl of culture medium were added immediately after the electroporation in the cuvette and the total solution was then transferred in 0.04% gelatin coated T25 flasks containing 5ml of reprogramming medium the contents of which are mentioned in detail in paragraph 2.2.7.

An identical protocol was utilised for nucleofection of the individual four transcription factors in fibroblasts. Each nucleofection required $2x10^6$ cells and 1µg per factor (Ex-SOX2, Ex-OCT4, Ex-KLF4, Ex-cMYC) to a total of 4µg per transfection. 4µg of an Ex-GFP vector was utilised as a control. The Ex vectors are shown in Figure 16 and were purchased from GeneCopia Ink.

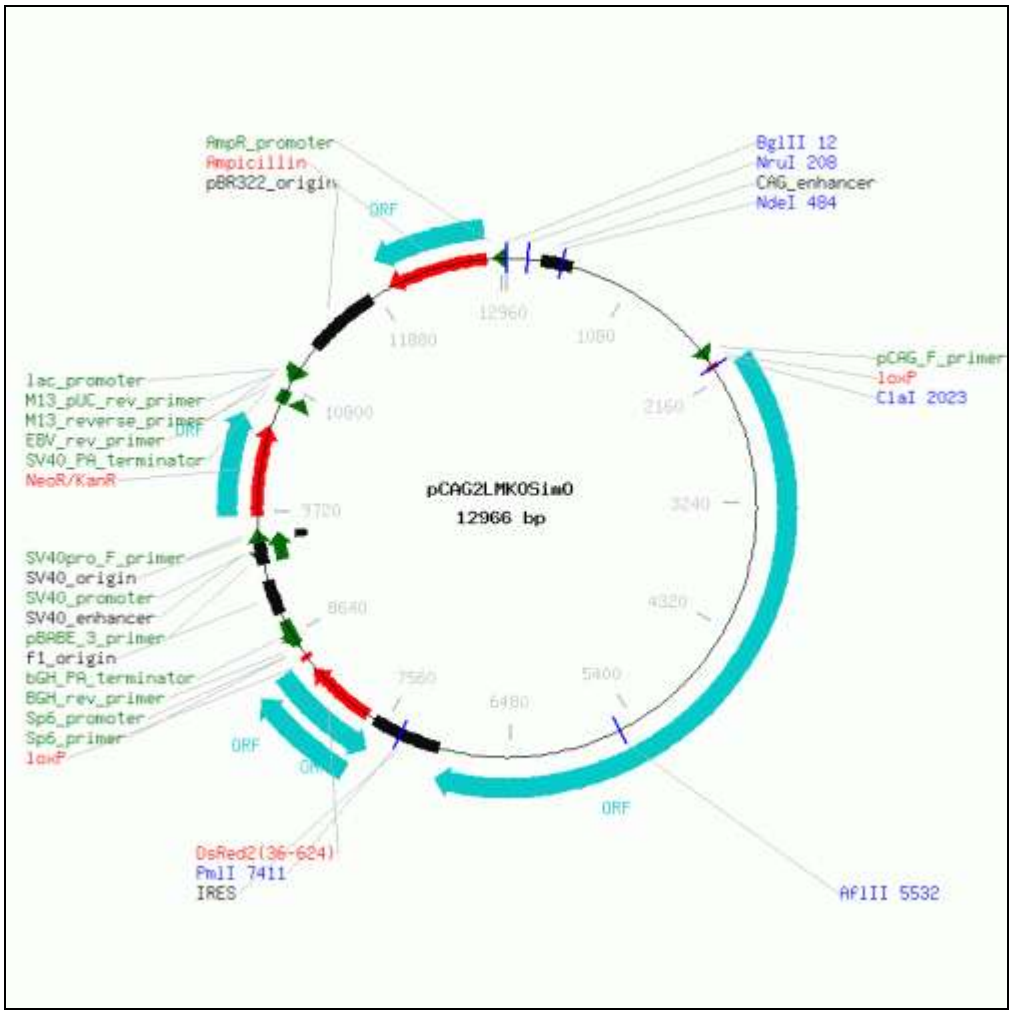
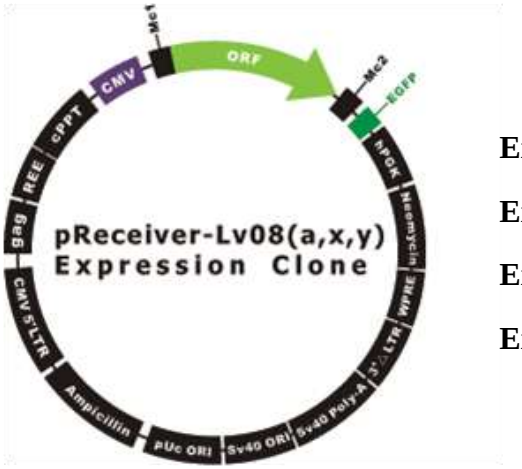


Figure 15. Map of the pCAG2LMKOSimO vector. Sox2, Oct4, Klf4 and cMyc coding regions are linked by three different 2A peptide sequences, F2A, T2A and E2A (reprogramming cassette) are transcribed from the CAG enhancer/promoter. The reprogramming cassette is followed by ires mOrange. The reprogramming cassette and ires mOrange are flanked by loxP sites (Kaji et al., 2009)



Ex-SOX2=945bp

Ex-OCT4=1083bp

Ex-KLF4=1413bp

Ex-cMYC=1365bp

Figure 16. Expression Clone (EX) construct map

2.2.6 Lentiviral induction of TetO-FUW-OSKM

A lentiviral construct containing all four factors in one vector was also utilised for induction of SOX2, OCT4, KLF4 and cMYC in human neonatal fibroblasts. An empty Teto-FUW vector generated as described above was used as control. The lentiviral particles were kindly generated by Dr. Andriana Margariti. ~500.000 cells were seeded on gelatine coated dishes 24 hours prior to infection. The next day, fibroblasts were incubated with 10^7 TU/ml Teto-FUW-OSKM or Teto-ctl in complete reprogramming medium supplemented with 10μg/ml of polybrene for 16-24 hours. Subsequently, fresh medium was added to the cells and the dishes remained in the incubator under the aforementioned conditions for an additional 3 days as part of the PiPS generation.

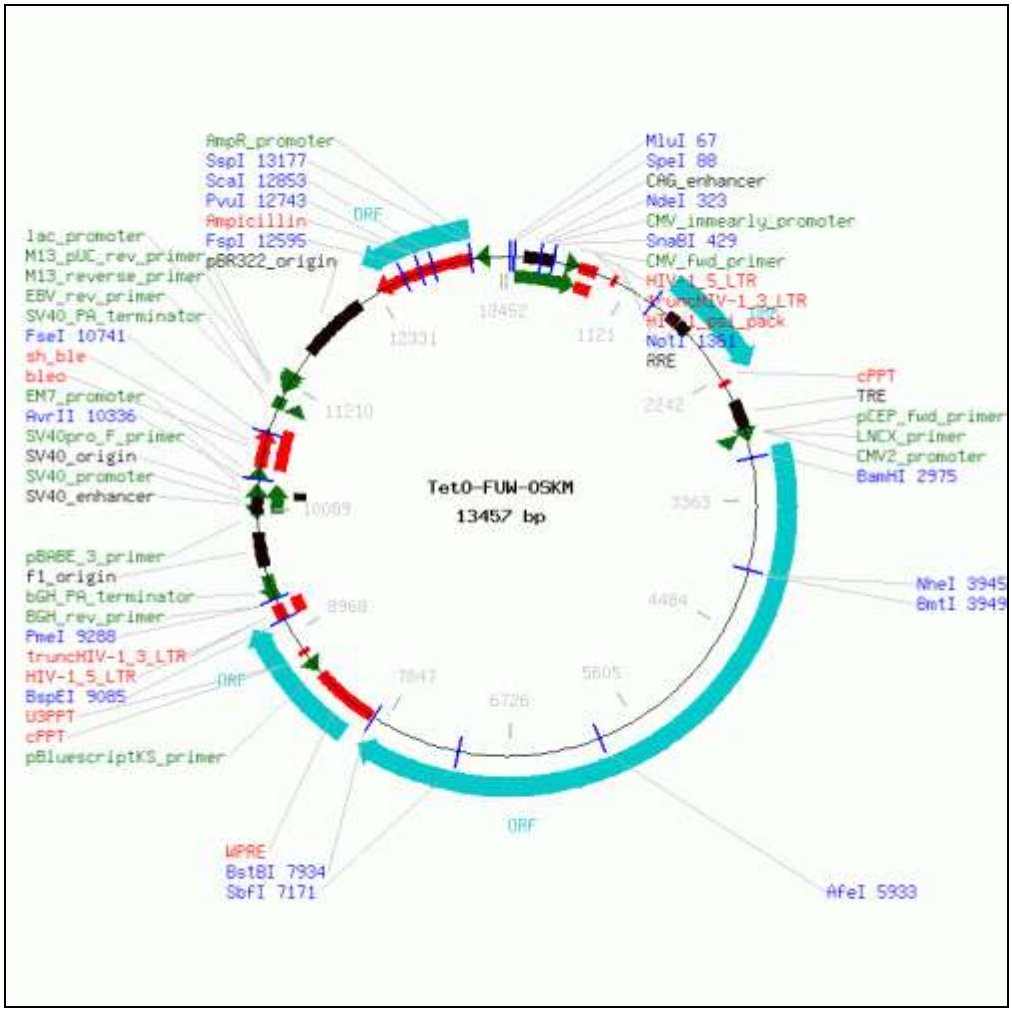


Figure 17. Map of the Teto-FUW-OSKM vector. This construct comprises a four-factor 2A (4F2A) doxycycline (DOX)-inducible lentivirus encoding mouse cDNAs for Oct4, Sox2, Klf4 and cMyc separated by three different 2A peptides (P2A, T2A, and E2A, respectively)(Carey et al., 2009).

2.2.7 Reprogramming

24 hours after nucleofection the reprogramming medium which comprised of Knockout DMEM medium optimized for embryonic stem cells (Life technologies (Gibco®) Cat. No. 12660-012), 20% Knockout Serum Replacement (Life technologies (Gibco®), Cat. No. 10828-028), 10ng/ml basic Fibroblast Growth Factor-2 (bFGF-2) (MACS Miltenyi Biotech Cat No. 130-093-837), 100 U/ml penicillin and streptomycin (Life technologies (Gibco®) Cat. No 15070-063), 0.2 mM L-glutamine (Life technologies (Life Technologies™) Cat. No. 25030-024), 0.1mM β-mercaptoethanol (Life technologies (Life Technologies™) Cat. No. 31350-010) and 0.1mM MEM Non Essential Amino Acids (Life technologies (Gibco®) Cat. No. 11140-050). The cells were maintained under these conditions for another 72 hours (in total 4 days after nucleofection) as part of the reprogramming process.

2.2.8 PiPS cell differentiation

PiPS cells were seeded on Collagen IV coated dishes (5µg/ml) and maintained in differentiation medium (DM) which contained MEM α (Life technologies (Gibco®) Cat. No. A10490-01) supplemented with 10% FBS (PAA Cat. No A15-151), 100U/ml penicillin and streptomycin (Life technologies (Gibco®) Cat. No. 15070-063), 0.2 mM L-glutamine (Life technologies (Life Technologies™) Cat. No. 25030-024), 0.1mM β-mercaptoethanol (Life technologies (Life Technologies™) Cat. No. 31350-010) and 10ng/ml Platelet Derived Growth Factor BB (PDGF-BB) (R&D systems Cat No: 220-BB). The cells were maintained under these conditions for 2, 3 and 4 days after which they were harvested and further analysed.

2.2.9 Harvesting cells

The supernatant medium was removed or collected from the flasks or dishes and cells were washed with cold PBS (4^{o}C). An additional volume (5-10ml) of cold PBS was added and cells were scraped off, descanted into 15ml tubes and centrifuged at 1000 rpm for 5 minutes. Supernatant PBS was discarded and cells were resuspended in 1ml of which 750µl and 250µl were transferred to micro centrifuge tubes and centrifuged shortly at 4^{o}C (high speed) for protein and RNA extraction respectively.

2.2.10 RNA extraction

RNA extraction was performed using RNeasy Mini Kit for isolation of total RNA from animal cells (Qiagen Cat. No 74106). It was performed according to the manufacturer's protocol at room temperature with a centrifugal force of at least 10.000 rpm unless otherwise stated. The cell pellet was resuspended in 350µl of RLT lysis buffer to disrupt the cell membrane and organelles and release the total RNA contained in the sample. The lysate was then transferred into a QIAshredder spin column and was centrifuged for 2 minutes to create a homogenous lysate by shearing high-molecular-weight genomic DNA and other cellular components. An equal volume of 70% ethanol was added to the homogenized lysate to provide appropriate binding conditions for the RNA to bind to the RNeasy spin column membrane. 700µl of the sample were applied to the RNeasy mini column and placed in a 2ml collection tube. The samples were centrifuged for 30 sec and the flow though obtained was discarded. 700µl of RW1 washing buffer provided by the kit was added to the column followed by centrifugation for 15 seconds.

The flow through was once again discarded and 2 more washes were carried out with 500µl of RPE buffer which was provided in the kit. The washing buffers are applied to make sure all contaminants are removed. The flow through was discarded and an additional centrifugation of 1 minute took place to remove all remaining solution that could dilute the RNA extract. To elute the RNA, the RNeasy column was transferred to a clean 1.5ml autoclaved micro centrifuge collection tube and 25µl of RNase-free water was added to the RNeasy column membrane followed by spinning for 1 minute. The RNA concentration was measure by using a Nanodrop Spectrophotometer.

2.2.11 Reverse transcription (RT) of RNA to obtain cDNA

cDNA was synthesised from 1µg of total RNA for each RT reaction. RT was performed using the Improm-II reverse transcription system (Promega Cat. No A3800) in a 20µl reaction outlined below. Mix A was heated at 70^{o}C for 5 minutes to allow denaturation of RNA. The samples were briefly placed on ice before Mix B was added. After adding Mix B, the samples were kept at 25^{o}C for 5min and then at 42^{o}C for 1 hour and 30 minutes for extension to take place. Finally a temperature of 70^{o}C was applied in order for the reverse transcriptase enzyme to be deactivated (Table 1). The cDNA obtained was diluted with 80µl of DEPC-treated water to obtain a final concentration of 10ng/µl.

Component		Volume (µl)	Final concentration
MIX A	Random primers	0.1	0.5 µg
	RNA	X	1 µg
	DEPC water	to 10	-
MIX B	Improm II 5x reaction buffer	4	1x
	25mM $MgCl_2$	2.4	3mM
	25mM dNTPs	0.8	1mM
	RNasin ribonuclease inhibitor	1	40U/µl
	Improm II RT enzyme	1	1U/µl
	DEPC-treated water	0.8	-

Table 1. Reverse transcription reaction solutions and concentrations

2.2.12 Conventional polymerase chain reaction (PCR)

50ng of cDNA were used to perform polymerase chain reactions using recombinant Taq DNA Polymerase (Invitrogen, 10342-53). The reaction mix and primer parameters are given below (Table 2).

Component	Volume (µl)	Final concentration
cDNA	5	50ng
10x PCR Buffer	2.5	1x
25mM dNTPs	0.2	0.17mM
50mM $MgCl_2$	1.2	2mM
10µM Forward Primer	1	0.7mM
10µM Reverse Primer	1	0.7mM
Taq DNA Polymerase	0.2	0.1 U
DEPC-treated water	13.9	-

Table 2. PCR solutions and concentrations

Primers specific to the PCR template were designed from human mRNA sequences using the Primer-BLAST tool (www.ncbi.nlm.gov.uk/tools/primer-blast). All primers were ordered from Sigma-Aldrich, resuspended in 1mM EDTA and the stocks (100mM) were stored at -80 ^{o}C. Working solutions of 10mM were kept at -20 ^{o}C.

	Sequence (5'→3')	Start Position	End Position	Annealing Temperature (°C)	Cycles	Product Size (bp)
SMA Forward SMA Reverse	ACTGGGACGACATGGAAAAG GAAGGAATAGCCACGCTCAG	714 1081	733 1062	55	28	368
Calponin Forward Calponin Reverse	AGGCTCCGTGAAGAAGATCA CCACGTTCACCTTGTTTCCT	371 585	390 566	58	30	215
SM22a Forward SM22a Reverse	AACAGCCTGTACCCTGATGG CGGTAGTGCCCATCATTCTT	717 955	736 936	58	30	239
Myocardin Forward Myocardin Reverse	AGCAGACAGCAGTCATGGTG CTTGTTCAAAGGAAGCCGAG	2438 2805	2457 2786	58	30	368
TGFβi Forward TGFβi Reverse	GACCCTTCGGGCTGCTGTGG GCCCACCTCCAGTGTCGTGC	923 1175	942 1156	58	30	253
Anexin3 Forward Anexin3 Reverse	GCACAGCGGCAGCTGATTGT TCCCGCGCCCTTCATGGATT	461 631	480 612	58	30	171
TEK Forward TEK Reverse	GGCCCAGGGGGAATGGAGTG CCGAGGTCCGCTGGTGCTTG	2302 2667	2321 2648	58	30	366
MPZL2 Forward MPZL2 Reverse	CCAGCTTTGCCCCTGTGGGT AGTGTGCACGACGCTGAGCC	290 576	309 557	58	30	287
NDUFA4L2 Forward NDUFA4L2 Reverse	GCAGGAGCCAGTCTTGGGGC TGTCCCAGCAGACGTCGGGG	268 418	287 399	58	30	151

Gene Target	Sequence (5' → 3')	Start Position	End Position	Annealing Temperature (°C)	Cycles	Product Size (bp)
BIK BCL2 Forward BIK BCL2 Reverse	AGCTCCTGGAACCCCCGACC TCGCAGGACACCCAGGACCC	119 461	138 442	58	30	343
STMN2 Forward STMN2 Reverse	CGTGCCTCTGGCCAGGCTTT CTCCGCAGCATGCCTCTCCTTT	193 549	212 528	58	30	357
DKK3 Forward DKK3 Reverse	CCATGCCGGGGCCAGAGGAT CACCAGGCTGTGGCTGTGGG	733 1068	752 1049	58	30	336
b actin Forward b actin Reverse	TGGCACCACACCTTCTACAATGAGC GCACAGCTTCTCCTTAATGTCACGC	340	735	58	28	395
SOX2 Forward SOX2 Reverse	CAGCCCGGACCGCGTCAAGCG GTGCTCCTTCATGTGCAGCGC	545	745	58	30	200
OCT4 Forward OCT4 Reverse	CAGGAGTCGGGGTGGAGAGCA GCCTCAAAGCGGCAGATGGTC	350	620	58	30	270
KLF4 Forward KLF4 Reverse	CATCAACGACGTGAGCCCCTC CCTGCTTGATCTTGGGGCACG	1161	1432	58	30	270
cMYC Forward cMYC Reverse	CACTGGTCCTCAAGAGGTGCC CTCTGCTTGGACGGACAGGAT	1451	1797	58	30	350

Table 3. Conventional primer sequences and their parameters

The PCR protocols were optimized: Initial denaturation, 95^{o}C for 5 minutes; 30-35 cycles of 94 ^{o}C for 30 seconds, 58 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute; Final extension 72 ^{o}C for 10 minutes

2.2.13 Quantitative real time polymerase reaction (Q-PCR)

Relative gene expression was detected by Q-PCR using the Eppendorf Mastercycler® ep realplex. 20ng of cDNA were combined with 10µl of SYBR Green (Qiagen, Cat. No. 204057) and combined forward and reverse primers for the target gene in a final concentration of 0.75µM. The final volume was adjusted to 20µl per sample with DEPC water and the samples were loaded on 96 well plates (Eppendorf, twin.tec real-time PCR plates). The Q-PCR conditions were 5min at 95^{o}C and then 40 cyles of 95 ^{o}C for 15 seconds and 60^{o}C for 30 seconds followed by 10 minutes of 95^{o}C to establish the melting curve of the primers. The threshold cycle (Ct) values were automatically obtained in excel format and the 18s RNA Ct values served as the internal endogenous control. For every sample, Q-PCR was performed in duplicate. The primers used for Q-PCR were designed using a software provided by DNA Integrated Technologies (IDT) (http://eu.idtdna.com/scitools/Applications/RealTimePCR/) and are shown below (Table 4).

Gene Target	Sequence (5' → 3')	Start Position	End Position	Product Size (bp)
SOX2 Forward SOX2 Reverse	CACATGAAGGAGCACCCGGATTAT TCCGGGAAGCGTGTACTTATCCTT	307	393	87
OCT4 Forward OCT4 Reverse	AGCTGCTGAAGCAGAAGAGGATCA TCTCATTGTTGTCGGCTTCCTCCA	431	625	195
KLF4 Forward KLF4 Reverse	ACAGCCACCCACACTTGTGACTAT AGTGGTAAGGTTTCTCGCCTGTGT	1189	1294	106
cMYC Forward cMYC Reverse	TGGTGTCTGTGGAGAAGAGGCAAA TTGGCAGCTGGATAGTCCTTCCTT	891	1068	178
SMA Forward SMA Reverse	TGACAATGGCTCTGGGCTCTGTAA TTCGTCACCCACGTAGCTGTCTTT	36	177	142
Calponin Forward Calponin Reverse	TTGAGGCCAACGACCTGTTTGAGA TCGAATTTCCGCTCCTGCTTCTCT	317	458	142
SM22 Forward SM22 Reverse	TTGAAGGCAAAGACATGGCAGCAG TCCACGGTAGTGCCCATCATTCTT	353	441	89
SMMHC Forward SMMHC Reverse	AGAAGCCAGGGAGAAGGAAACCAA TGGAGCTGACCAGGTCTTCCATTT	4449	4579	131
SRF Forward SRF Reverse	TGAGTGCCACTGGCTTTGAAGAGA AGAGGTGCTAGGTGCTGTTTGGAT	707	858	152
Myocardin Forward Myocardin Reverse	TTGAAAGCGGAGAAATGCCAGCAG ACTGTCGGTGGCATAGGGATCAAA	2358	2553	176
DKK3 Forward DKK3 Reverse	AGGTTGAGGAACTGATGGAGGACA ACCTTCGTGTCTGTGTTGGTCTCA	158	311	154
β catenin Forward β catenin Reverse	TGCAGTTCGCCTTCACTATGGACT GATTTGCGGGACAAAGGGCAAGAT	1449	1570	122

Gene Target	Sequence (5' → 3')	Start Position	End Position	Product Size (bp)
Axin 2 Forward Axin 2 Reverse	ACAACAGCATTGTCTCCAAGCAGC GCGCCTGGTCAAACATGATGGAAT	422	523	102
TCF1 Forward TCF1 Reverse	AATAGGGCGGAATGCATCCAGAGA TTGGCAAACCAGTTGTAGACACGC	709	809	101
18s Forward 18s Reverse	CCAGAGCGAAAGCATTTGCCAAGA TCGGCATCGTTTATGGTCGGAACT	988	1093	106
TEK Forward TEK Reverse	GAACTGCACACGTTTGGCAGAACT ACCTAAGCTTACAATCTGGCCCGT	766	943	178
HBA2 Forward HBA2 Reverse	ACGCTGGCGAGTATGGTGCG GGTCACCAGCAGGCAGTGGC	128	393	266
CD34 Forward CD34 Reverse	CACTGAGCAAGATGTTGCAAGCCA TCAGGAAATAGCCAGTGATGCCCA	1083	1192	110
CD133 Forward CD133 Reverse	TACCAAGGACAAGGCGTTCACAGA GTGCAAGCTCTTCAAGGTGCTGTT	1155	1355	200
cKIT Forward cKIT Reverse	TGAATGGCATGCTCCAATGTGTGG ACATCCACTGGCAGTACAGAAGCA	1352	1463	111
KDR Forward KDR Reverse	ATCCAGTGGGCTGATGACCAAGAA ACCAGAGATTCCATGCCACTTCCA	1229	1321	92

Table 4. Q-PCR primer sequences

2.2.14 Protein extraction

The cell pellet was resuspended in 30-50µl of a protein lysis buffer. The lysate was then sonicated with the Branson Sonifier 150 at the lowest setting for 12 sec at 4^{o}C and incubated on ice for at least 30 minutes. The lysate was then centrifuged at 13.000 rpm for 10 min at 4^{o}C. The supernatant was transferred to a new micro-centrifuge tube and the protein level was detected using a Biorad Protein Assay. 2µl of the protein lysate was mixed with 998µl of the Bio-Rad Reagent (1:5) and incubated at room temperature for 5 minutes. Duplicates were measured using the Bio-Rad Spectrophotometer 3000. 2µl of lysis buffer was used as the blank measurement.

2.2.15 Immunoblotting

25-50µg of protein were boiled in 1xSDS loading buffer for 10 minutes before loading onto NuPage®, 4-12% Bis-Tris gel immersed in NuPage® MOPS SDS running buffer in a XCell *SureLock*™ Mini-Cell (Life technologies (Novex®) Cat. No. NP0335BOX). In the case of supernatants, 20µl of medium were boiled with 1xSDS and prepared as described above. Protein ladder (Precision Plus Protein Ladder, Bio-Rad) was loaded simultaneously and the samples were run at 160V. The gel was then transferred onto a PVDF membrane (Amersham, hypond-P) with the XCell™ Blot Module (Invitrogen) at 30V for 2 hours and 30 minutes immersed in transfer buffer. The membrane was blocked with 5% milk in PBS-Tween containing 0.02% sodium azide, for 1 hour at room temperature and then incubated in the primary antibody solutions overnight at 4^{o}C. The antibodies used are listed in Table 5 and a representative blot of antibody specificity is shown in Figure S8.

Secondary antibodies diluted in 5% milk PBS-Tween (1:3000) were incubated in room temperature for 1 hour after washing with PBS-Tween (10minutes x3). Further washing was then carried out before addition of ECL detection solutions (3min). Exposure of films (Amersham, Kodak) was carried out using the Compact X4 (Xograph Imaging System).

2.2.16 Indirect Immunofluorescence Assay (IIFA)

Cells were trypsinized and resuspended in medium and ~10.000 cells were seeded on an 8-chamber slide coated with Collagen IV. The staining took place at least 24 hours after seeding. Medium was discarded and cells were washed twice with warm PBS. The cells were then fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes in room temperature. They were then washed x3 with cold PBS and permeabilized with 0.1% Triton-X-100 in PBS for 10 minutes in room temperature. An additional wash with cold PBS took place in room temperature for 5 minutes. For the frozen sections of vessels harvested from the ex-vivo and in-vivo experiments, the samples were fixed in 100% acetone for 10min and then washed x3 with PBS. All slides were then blocked with 5% normal swine serum in PBS and incubated for 30 minutes in room temperature. The primary antibodies used and the dilutions in which they were used are shown in Table 5. The samples were once again washed with cold PBS (10minutes x3) and secondary antibodies were applied in the concentrations shown in Table 5 for 30 minutes at 37 ^{o}C. Samples were washed 3 x 5 minutes before counterstaining with DAPI (1:1000 in PBS) for 2 minutes at room temperature. They were then mounted with fluorescent mounting media (Dako) and images were taken with the Axio Imager.M2 microscope and AxioVision Digital Imaging System (Carl Zeiss Ltd) or SP5 confocal microscopy.

2.2.17 Quantification of fluorescence area by Volocity

Images for figures 39, S1 and S6 were captured with a 20x dry objective on an Olympus IX81 wide field inverted microscope with the use of the imaging program Volocity. For every set of experiments figures were captured at the same levels of exposure, gain and offset. Quantification of fluorescence was then performed through Volocity. For every set of experiments a threshold of fluorescence of SD+1 of the image mean was set between ctl and treated sample and fluorescence was calculated as total fluorescence area divided by the total number of cells. For example Sum of Calponin area per number of nuclei (μm^2/ cell).

Antibody	W.B	IIFA	Secondaries for IIFA	Origin and Cat. No
SMA	1:5000	1:200	Anti-Mouse conjugated to FITC	Sigma A5228
SMMHC	-	1:100		Abcam Ab683
Calponin	1:10.000	1:200	Anti-Rabbit conjugated to FITC	Abcam 46794
SM22	1:2000	1:200		Abcam 14106
β catenin	1:200	1:50		Santa Cruz sc-7199
GAPDH	1:200	-	-	Santa Cruz Sc-25778
GSK3	1:1000	-		Cell signalling 5676
DKK3	1:1000	1:50	Anti-Goat conjugated to TRITC	R&D AF1118
Kremen 1	1:1000	-	-	R&D AF1647
A-Fetoprotein	-	1:50	Anti-Mouse conjugated to FITC	R&D MAB1368
β-tubulin III	-	1:50		R&D MAB1195

Table 5. Antibodies used for immunoblotting and IIFA

2.2.18 Transient overexpression of DKK3 in PiPS-SMCs

PiPS-SMCs were trypsinized and counted, and $2x10^6$ cells were aliquoted in 15ml tubes and centrifuged at 1000 rpm for 5 minutes. They were then nucleofected with 2µg of DKK3 plasmid (Figure 18) (Addgene, plasmid 15496 (Krupnik et al., 1999)) as described in paragraph 2.2.5. A pCMV5 vector (Margariti et al., 2009) was also nucleofected in PiPS-SMCs as a mock control. The cells were then seeded on Collagen IV coated plates (5µg/ml) and harvested 48 hours later. The medium was refreshed 24 hours after nucleofection.

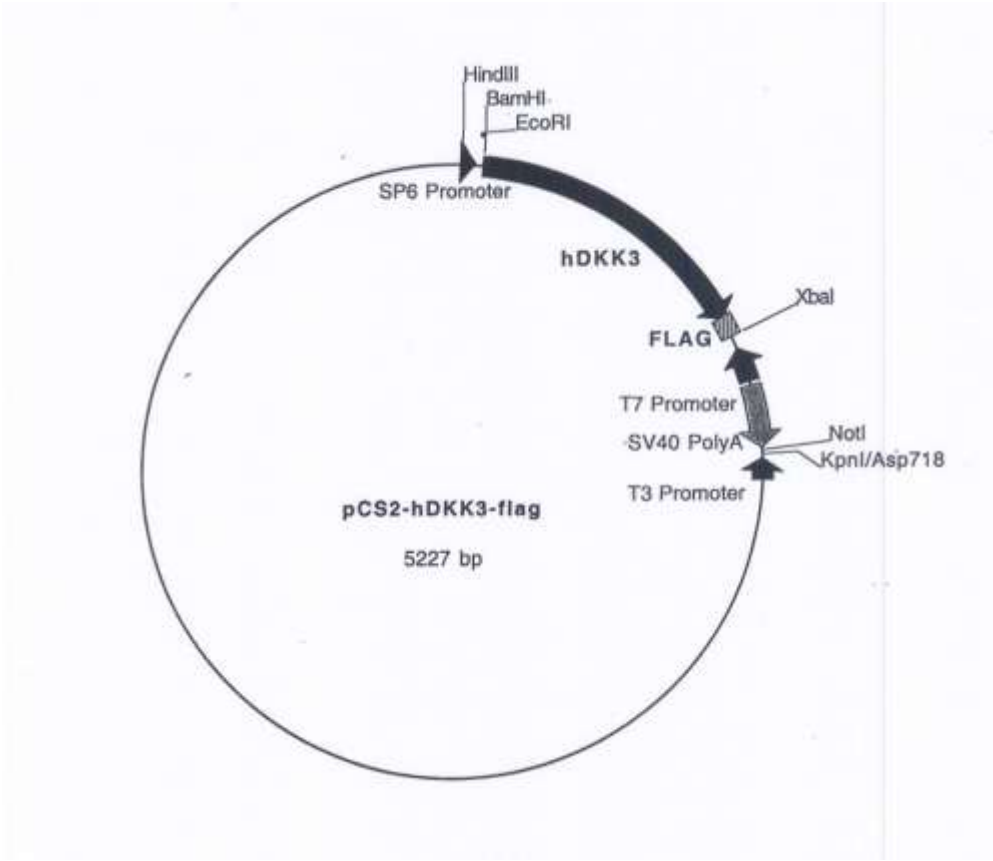


Figure 18. Map of the pCS2-hDKK3-Flag vector

2.2.19 Lentiviral particle generation

For the generation of Lentiviral particles, 293-T cells were transfected with the lentiviral vector and the packaging plasmids (obtained by Genecopoeia) according to the protocol provided. The supernatant containing the lentivirus was harvested 48 hours later, filtered, and the transduction unit (TU) was calculated, as previously described (Margariti et al., 2010). The non-targeting shRNA vector was used to generate the control particles.

2.2.20 DKK3 shRNA lentivirus transduction

For lentiviral infection, 500.000 PiPS-SMCs were seeded on Collagen IV coated plates and left to adhere overnight. The next day, cells were incubated with shDKK3 or the non targeting control (10^7TU/ml) complete growth medium supplemented with 10μg/ml of polybrene for 16-24 hours. Subsequently, fresh serum free medium was added to the cells and the plates were returned to the incubator and harvested 24 or 48 hours later. The shDKK3 lentivirus was kindly generated by Dr. Andriana Margariti.

2.2.21 Enzyme-linked immunosorbent assay (ELISA)

Supernatant was collected from the cells, aliquoted and stored at -80 ^{o}C until used. The concentration of the DKK3 released glycoprotein in the supernatant was detected by an R&D DKK3 ELISA kit (R&D, Cat. No. DY1118). The capture antibody was diluted to working concentration in PBS without carrier protein. A 96-well microplate was immediately coated with 100μl per well of the diluted Capture antibody and was sealed and incubated overnight at room temperature. The solution was then removed and the wells were washed x3 with Wash Buffer (0.05% Tween® 20 in PBS). Each well was then blocked with 300μl of Reagent Diluent (1% BSA in PBS) and maintained in room temperature for at least 1 hour. The wells were once again washed with Wash Buffer x3.

100µl of the supernatant sample or standards in Reagent Diluent were added per well (Standards comprised human recombinant DKK3 in 7 2-fold serial dilutions with 2000pg peak and were used for the generation of a standard curve). The plate was covered with an adhesive strip and incubated at room temperature for 2 hours. Each well was washed 3x with Wash buffer. 100µl of Detection antibody (working concentration of 200ng/ml in Reagent Diluent) were added in per well, the plate was covered again with an adhesive strip and incubated at room temperature for 2 hours. 100µl of Streptavidin-HRP were added per well following x3 washes with Wash Buffer and incubated at room temperature for 20 minutes avoiding direct exposure to light. The wells were once again washed x3 and 100µl of Substrate Solution (1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine)) were added per well and incubated at room temperature for another 20 minutes. Finally, the reaction was stopped using 50µl of Stop Solution (2N H2SO4). The optical density of each well was determined immediately using a Teecan microplate reader set to 450 nm. Wavelength correction was set to 540 nm and was used for normalisation of the readings. A standard curve was generated and the concentration of release DKK3 glycoprotein of each sample was calculated in ng/ml.

2.2.22 BrdU Assay

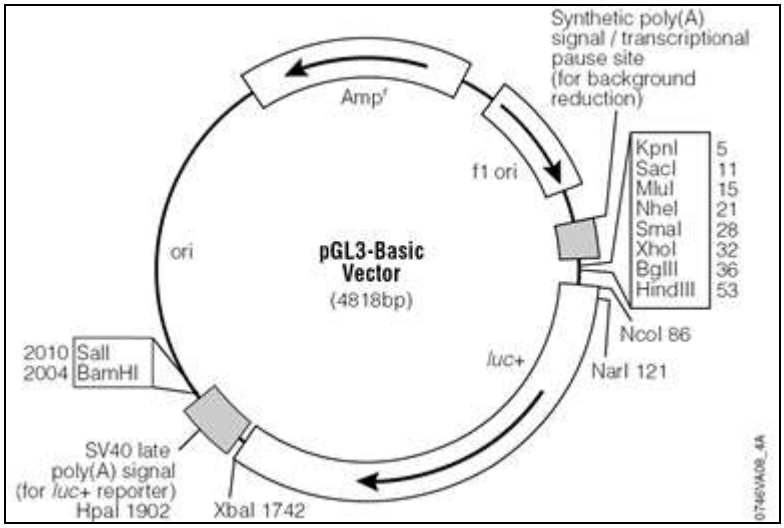
PiPS cells were differentiated for 2 days and then nucleofected with the DKK3 plasmid or with an empty pCMV5 vector as described in paragraph 2.2.18. They were then seeded on Collagen IV coated 12 well plates and Cell Proliferation ELISA, BrdU (colorimetric) (Roche Cat. No. 11647229001) was performed as according to the provided protocol. The optical density of each well was determined by using a Teecan microplate reader set to 450 nm. Wavelength correction was set to 690 nm and was used for normalisation of the readings.

2.2.23. Treatment with human recombinant DKK3 cytokine

PiPS cells were seeded on Collagen IV for differentiation for 2 days. The medium was removed and refreshed with serum free medium in which the cells were maintained overnight. The following day the cells were stimulated with 50ng/ml human recombinant DKK3 cytokine (R&D, Cat. No. 1118DK) overnight or for 6 hours for the β catenin translocation and co-immunoprecipitation experiments as described next.

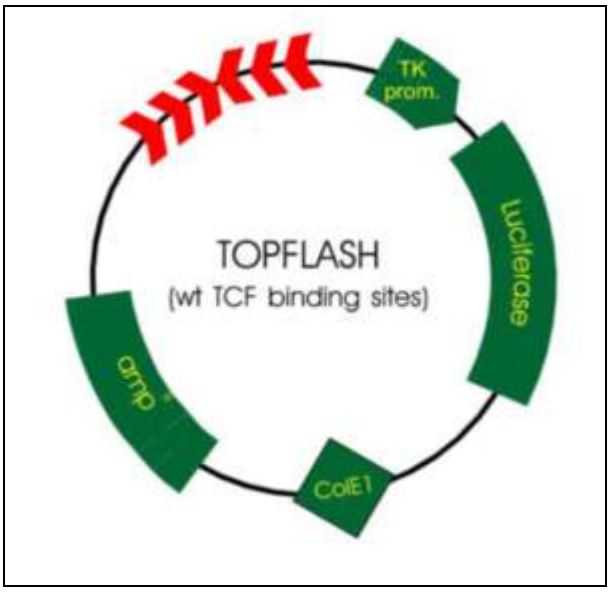
2.2.24 Transient transfection and Luciferase Reporter Assay

For the Luciferase Reporter Assays, ~10.000 PiPS cells were seeded per well in Collagen IV coated 12-well plates. 48 hours later, transfections of the reporter plasmids pGL3-Luc-SM22 (Margariti et al., 2009) (Figure 19) and TopFlash (Milipore, Cat. No. 21-170) (Figure 20) (0.33μg/well) together with the DKK3 expression vector (paragraph 2.2.16 and Figure 18) (0.16μg/well) were set up using FuGene®6 reagent (Promega, Cat. No. E2691) as instructed by the manufacturer. pGL3-Luc-Renilla (0.1μg/ well) was also included in all transfections as an internal control and a pCMV5 vector was also used as a mock control. The Luciferase (Luciferase Assay System, Promega, Cat. No. E1501) and Renilla (Coelenterazine, Promega, Cat. No. S200A) enzymatic activities were detected 48 hours after transfection using the Lumat LB 9507 illuminometer. Relative luciferase unit (RLU) was defined as the ratio of luciferase activity to Renilla activity with that of control set as 1.0. For verification of the DKK3 expression vector, HEK293-T cells (Immortalized Human Kidney Cells, ATCC) were transfected with 2μg per T75 flask at 50-60% confluence with FuGene®6 followed by immunoblotting (data not shown).



(Promega)

Figure 19. Map of the pGL3-Luc-Basic vector



(Millipore)

Figure 20. Map of the TopFlash reporter vector

2.2.25 DKK3 shRNA and β catenin shRNA lentiviral transduction and Luciferase Reporter Assay

~10.000 PiPS cells per well were seeded on Collagen IV coated 12-well plates. 24 hours later the cells were virally transduced with DKK3 shRNA or β catenin shRNA as previously described and were returned to the incubator for 24 hours. Cells were also infected with non targeting shRNA which comprised the control sample. 24 after infection with the DKK3 shRNA, the cells were transiently transfected with pGL3-Luc-SM22 or TopFlash vectors (0.33μg/well) simultaneously with pGL3-Luc-Renilla (0.1μg/well) while the β catenin shRNA infected cells were transiently transfected with pGL3-Luc-SM22 (0.33μg/well) together with the DKK3 expression vector (0.16μg/well) and the pGL3-Luc-Renilla (0.33μg/well). A pCMV5 vector was used as a mock control. The samples were harvested 48 hours after transfection and the Luciferase and Renilla enzymatic activity was measure as described in paragraph 2.2.22. Verifications of suppression of DKK3 and β catenin were performed by lentiviral transduction of PiPS-SMCs or PiPS-SMCs overexpression DKK3 respectively followed by Q-PCR and western blot analysis.

2.2.26 Co-immunoprecipitation

HEK293-T cells were seeded in T75 flasks 24 hours prior to transient transfection in a number sufficient to reach 50-60% confluence the following day. One hour prior to transfection the medium was refreshed and the cells were transfected with 4μg of a DKK3 expression or a pCMV5 vector (mock control) using the FuGene®6 Reagent. 48 hours later the cells were harvested and lysed by 1 hour rotation at 4^{o}C. For the PiPS-SMC experiments, PiPS or ctl cells were seeded on Collagen IV coated T75 flasks where they were differentiated for 3 days.

The medium of a portion of the 3 day differentiated PiPS-SMCs was refreshed to serum free medium overnight while the rest were refreshed with complete medium as per usual. The following day the serum starved cells were stimulated with 50ng/ml human recombinant DKK3 for 6 hours and subsequently all the samples were harvested and lysed by 1 hour rotation at 4^{o}C. 200µg to 1mg of whole cell lysate were subjected to a standard co-immunoprecipitation procedure. Lysates were pre-cleared with normal rabbit IgG and then incubated with a rabbit DKK3 antibody (Santa Cruz, sc-25518) (0.2 to 1µg) for 2 hours at 4^{o}C and precipitated for a further 2 hours with protein G beads (EZview™ Red Protein G Affinity Gel, Sigma, Cat. No. E3403). The bound proteins were then eluted with 20µl 2 x SDS-sample elution buffer and 5µl of 6x Loading Buffer by boiling for 30 minutes at 70^{o}C. The samples were then centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred in new microcentrifuge tubes. They were then resolved by SDS gel electrophoresis simultaneously with 50µg of unprecipitated lysate which comprised the input samples and analysed by immunoblotting with Kremen 1 and DKK3 antibodies (both Goat raised).

2.2.27 Deglycosylation and Luciferase Reporter Assay

PiPS-SMCs were seeded in 12-well plates and transiently transfected with reporter (pGL3-Luc-SM22 TopFlash) (0.33µg/well) and Renilla (pGL3-Luc-Renilla) vectors (0.1µg/well) as previously described. The cells were then treated with PNGase F from Elizabethkingia miricola (Sigma, Cat. No. P7367) for 6 hours in a final concentration of 1U/ml. Cells were also treated with inactivated PNGase F after inactivation of the enzyme at 90^{o}C for 15min as a control.

The samples were then harvested and the Luciferase and Renilla enzymatic activity was detected as previously described. Successful deglycosylation of DKK3 was assessed by immunoblotting of supernatants with DKK3 (data not shown).

2.2.28 Selection of PiPS cells

Human neonatal fibroblasts were nucleofected with the polycistronic OSKM plasmid containing all four factors as described in paragraph 2.2.5. Neomycin selection (Sigma G418, Cat. No. G8168) at a concentration of 25 μg/ml, was initiated 24 hours after the nucleofection and continued up to day 4 when a pure population of PiPS cells expressing an mOrange marker as well as the four transcription factors was obtained. The cells were used for *ex vivo* applications in a specially constructed bioreactor as described in the following paragraph. In all the other experiments the cells were not selected in order to obtain a sufficient number of cells to perform further analysis.

2.2.29 Cell seeding and vascular bioreactor

PiPSs or fibroblasts were seeded on aortic grafts, which were previously decellularized (with sodium dodecyl sulfate (SDS) at 0.075 % and washed in PBS), in a special constructed bioreactor and sheer stress was applied. Briefly, a roller pump (Masterflex: Standard Drive, model 7520; Standard Pump Head, model 7018-20; Codane Tubing, Cole-Parmer UK) was used to produce mean flow in the bioreactor, the grafts were fixed between two 25 G needles after hosting them on plastic tubes fixed by 8-0 silk sutures (8-0 black virgin silk, Ethicon Inc., Johnson & Johnson, Norderstedt, Germany). The complete setup was maintained in a standard CO_2 incubator at 37 °C. The scaffolds were placed for the seeding process in a self constructed incubation chamber and

preconditioned with culture media for 2 hours. Then, 2 x 10^6 cells of PiPSs or fibroblasts in DM containing 25ng/ml PDGF-BB were seeded on the scaffolds via direct injection and allowed to seed for 12 hours before the initial flow was set up. Decellularized vessels have also been used as a second control. Shear stress was applied at stepwise rates ranging from 5 to 20 dynes/cm^2 over a period of 48 hours. After this time point no increase in the shear stress rate was conducted and the grafts remained under constant shear stress of 20 dynes/cm^2 until they were harvested by day 5. For the last 24 hours 100 Units of heparin per ml of total circulating reactor media volume (heparin sodium salt diluted, from porcine gastrointestinal mucosa, Sigma Aldrich) was added every 12 hours. For the double seeded PiPS cells tissue engineered vessels, decellularized vessel scaffolds were placed in a self constructed incubation chamber and preconditioned with DM medium containing 25ng/ml PDGF-BB for 2 hours. After this period 2 x 10^6 PiPS cells in DM/PDGF-BB were directly injected and allowed to seed for 12 hours at a continuous rotational movement before initial flow was set up. Shear stress was applied at 10 dynes/sqcm^2 over a period of 48 hours. Then, the circulating media was exchanged to EGM-2 media and a second seeding step was initiated with 1 x 10^6 PiPS cells and after another 12 hours seeding period the shear stress rate was stepwise adjusted up to 35 dynes/sqcm^2. The grafts remained under constant shear stress of 35 dynes/sqcm^2 and harvested on day 5.The engineered vessels were harvested and used for further analysis in vitro or immediately grafted to animals.

2.2.30 Statistical analysis

Data expressed as the ±SEM were analysed with a two-tailed student's *t*-test for 2 groups or pair wised comparisons using the Graphpad Prism v5.0 software. A value of $P<0.05$ was considered to be significant.

Buffer	Composition
2X SDS Sample Elution Buffer	4% sodium dodecyl sulphate, 20% glycerol, 0.04% bromophenol blue, 125mM Tris-HCl (ph6.8)
5X SDS Loading Buffer	10% sodium dodecyl sulphate, 50% glycerol, 0.05% bromophenol blue, 10mM β-mercaptoethanol in 500mM Tris-hydrochloride (pH 6.8)
6x DNA Loading Buffer	30% glycerol, 0.3% bromophenol blue in distilled water
IP-A	25mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA (pH 8.0), 1.0% Triton X, cocktail of protease inhibitors (Roche; 1 tablet in 50ml)
IP-B	25mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA (pH 8.0), cocktail of protease inhibitors (Roche; 1 tablet in 50ml)
Phosphate Buffered Saline	137 mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 • 2 H2O, 1.76mM KH_2PO_4 to pH 7.4
Protein Lysis Buffer	1mM EDTA, 50mM tris-hydochloride (pH7.4), 150mM NaCl, 1% Triton X, cocktail of protease inhibitors (Roche;1 tablet in 50ml)
Transfer Buffer	25mM Tris, 192mM glycine, 10% methanol in ddH_2O.

Table 6. Table of Buffers and their composition

Chapter 3
Results

3.1 Gene expression is altered during fibroblast cell reprogramming from as early as day four.

Over recent years human and mouse iPS cells have successfully been generated in our laboratory. However, the molecular mechanisms as well as the pathways regulating the transition of terminally differentiated cells into pluripotent cells have not yet been elucidated. In order to explore the molecular changes taking place during the complicated process of reprogramming, human neonatal fibroblasts were virally transduced to overexpress the four transcription factors SOX2, OCT4, KLF4 and cMYC. This introduction of the four factors was coupled with culturing in reprogramming media to initiate a standard reprogramming protocol (Figure 21A). Samples were then harvested at different time points (4, 7, 14 and 21 days) and subjected to microarray analysis. The results revealed that during the process of reprogramming, the expression of 198 genes was altered on day 4, 107 on day 7, 97 on day 14 and 131 on day 21 (Table S1). Interestingly, further analysis revealed that during the first four days of reprogramming, the expression of a greater number of genes involved in cell movement, proliferation, cellular growth and development were altered when compared to days 7, 14 and 21. Finally, the expression of genes associated to differentiation was found to be altered from as early as day 4 (Figure S1). The differential expression profile of a number of these genes was confirmed by conventional PCR (Figure 21B, result representative of three experiments). These results reveal that specific gene expression patterns are altered from as early as day 4 during somatic cell reprogramming.

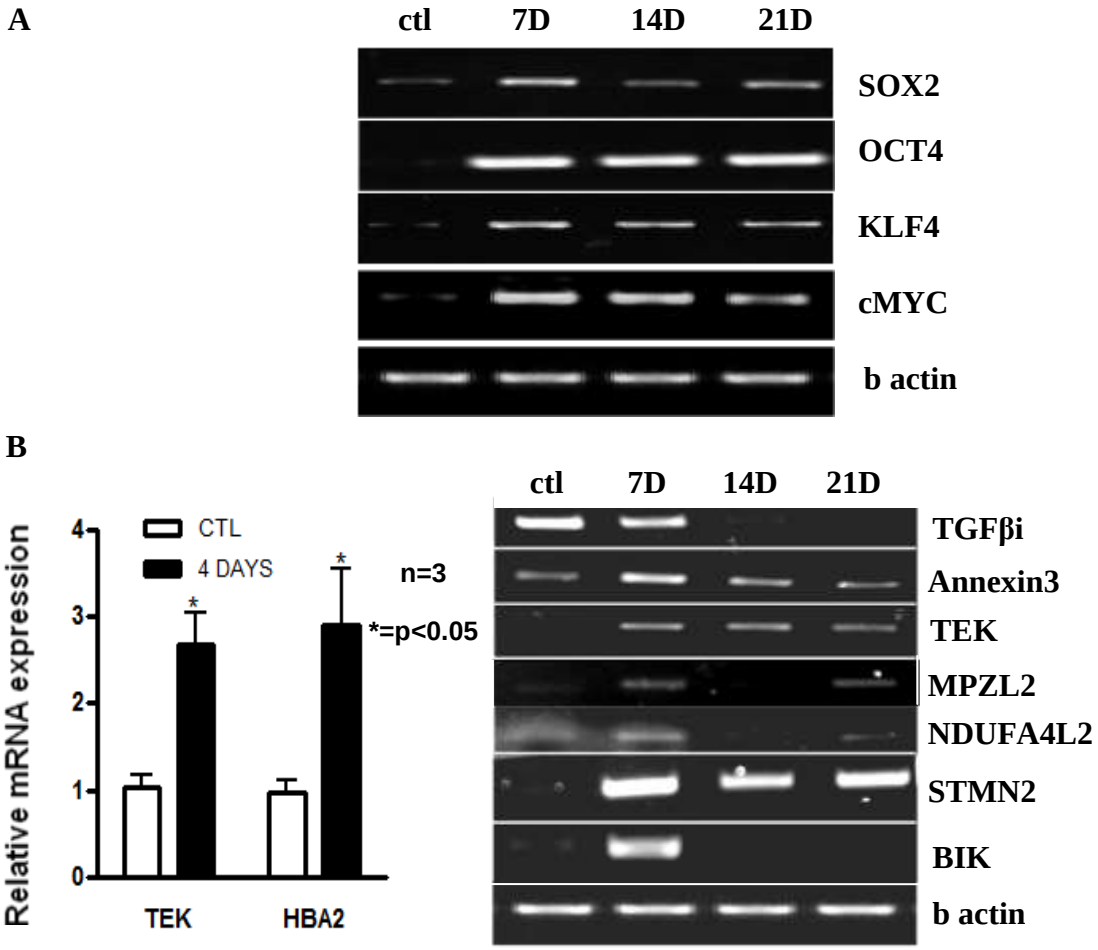


Figure 21: (A) Confirmation of induction of the four genes during the reprogramming, for the samples subjected to microarray analysis. Human embryonic fibroblasts were infected with lentiviral constructs encoding the four genes SOX2, OCT4, KLF4 and cMYC or a control construct (10^7TU/ml) and maintained in reprogramming medium for different timepoints. **(B) Confirmation of differential expression of selected genes from the microarray during the reprogramming process, using Q-PCR and conventional PCR.** TEK and HBA2 were found to be induced after 4 days (4D) (the mean ±SEM of 3 individual experiments is shown) and TGFBi was confirmed to be downregulated during all the time points. Anexxin3 was confirmed to only be upregulated during the first 7 days (7D) of reprogramming while STMN2 was significantly upregulated during all three time-points (images representative of 3 experiments).

3.2 Characterization of four day reprogrammed cells or Partially-iPS (PiPS)

Based on the result of the microarray analysis, where altered gene patterns appear very early during the reprogramming, four day reprogrammed cells were generated and then fully characterized. Human fibroblasts were nucleofected with a linearised pCAG2LMKOSimO plasmid encoding all four genes, by nucleofection with four plasmids each containing one of the genes (Ex-SOX2, Ex-OCT4, EX-KLF4 and Ex-cMYC) or by lentiviral infection with viruses encoding all four genes in one vector. They were then maintained in reprogramming media for four days and were defined as Partially-iPS (PiPS) cells. Simultaneously, fibroblasts were nucleofected or infected with empty vectors, they were maintained in the same culture conditions and were used as controls (ctl). Although PiPS cells did not form colonies at this stage of reprogramming, they displayed morphological differences when compared to ctl cells (Figure 22). PiPS cells expressed the four genes at both the mRNA (Figure 23A) and protein levels (Figure 23B). However, significant differences on the levels of expression of progenitor markers such as cKIT, CD34 and CD133 were not detected. Interestingly, PiPS cells expressed VEGFR2 (KDR) (Figure 24A), results which were also confirmed by FACS analysis (Figure 24B). Finally, PiPS cells were negative for the ESC marker alkaline phosphatase (Fig 25A) and did not form teratomas *in vivo* 2 months after subcutaneous transplantation in SCID mice (Figure 25B) as recently shown by Margariti *et al* (Margariti et al., 2012).

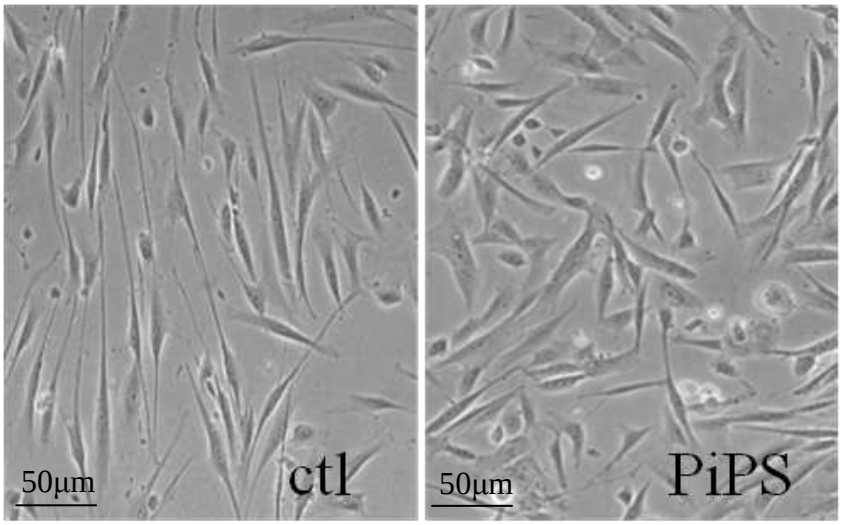
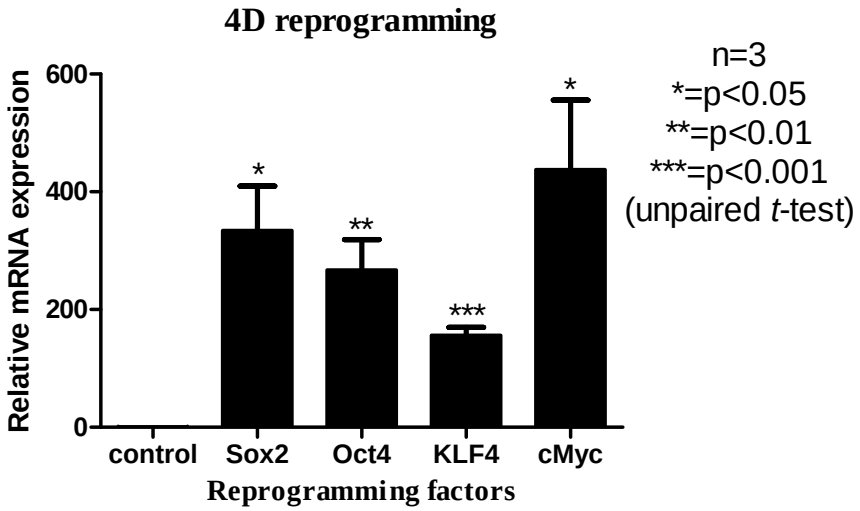


Figure 22. Morphological differences between Partially-iPS (PiPS) cells and control (ctl) cells. Human embryonic fibroblasts were nucleofected with a pCAG2LMKOSimO vector (4μg/2x10^6 cells) containing SOX2, OCT4, KLF4 and cMYC and were maintained in reprogramming conditions for four days while the ctl cells were nucleofected with the pCAG2L control vector (4μg/2x10^6 cells) and were also maintained in reprogramming media. Light microscope images reveal that the morphology of the PiPS cells is different than that of the ctl cells at this stage. Images were taken with the Nikon Eclipse TS100.

A



B

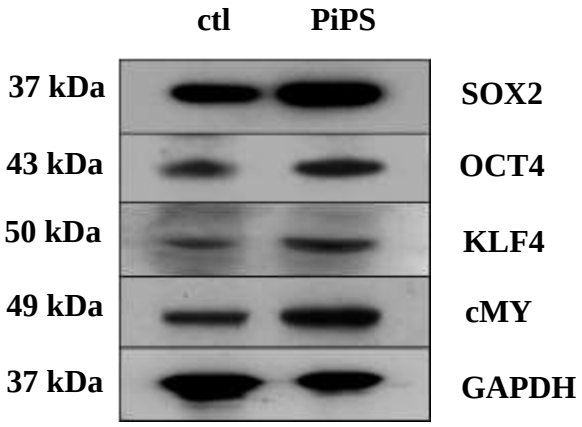
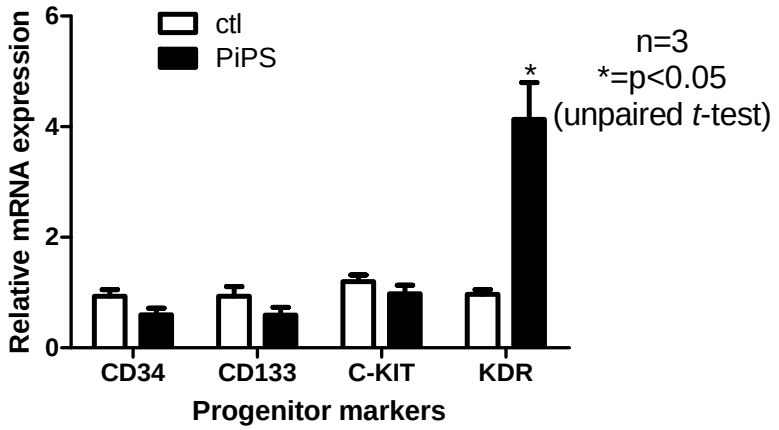


Figure 23. (A) Confirmation of overexpression of the four factors after 4 days of reprogramming at the mRNA level and (B) the protein level. Human embryonic fibroblasts were nucleofected with a pCAG2LMKOSimO vector (4µg/2x10^6 cells) containing SOX2, OCT4, KLF4 and cMYC and were maintained in reprogramming conditions for 4 days while the ctl cells were nucleofected with the pCAG2L control vector (4µg/2x10^6 cells) and were also maintained in reprogramming media. Samples were harvested 4 days later and subjected to Q-PCR (the mean ±SEM of 3 individual experiments is shown) and western blot analysis respectively (data representative of 3 experiments)**.**

A



B

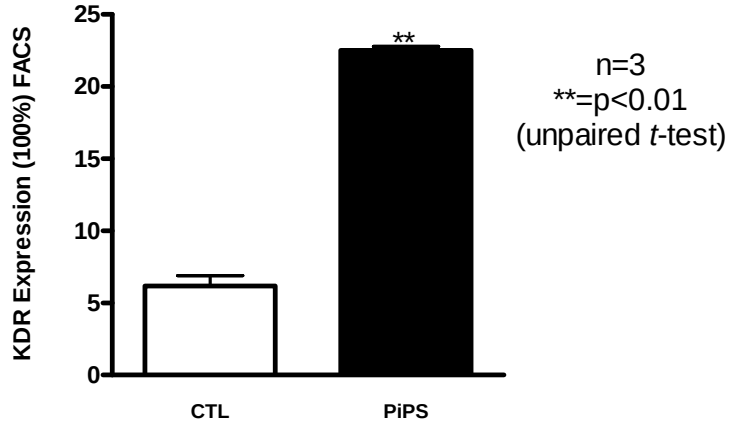


Figure 24. (A)VEGFR2 (KDR) is upregulated in PiPS cells after 4 days of reprogramming. Human fibroblasts were reprogrammed for four days and the mRNA levels of the progenitor markers CD34, CD133, C-KIT and KDR were assessed by Q-PCR. KDR was found to be upregulated in PiPS cells after four days of reprogramming when compared to the ctl cells (the mean ±SEM of 3 individual experiments is shown). **(B) FACS analysis confirmed the upregulation of KDR expression in PiPS cells** (mean ±SEM of 3 individual experiments) (Figure 24B from Margariti *et al*. submitted manuscript).

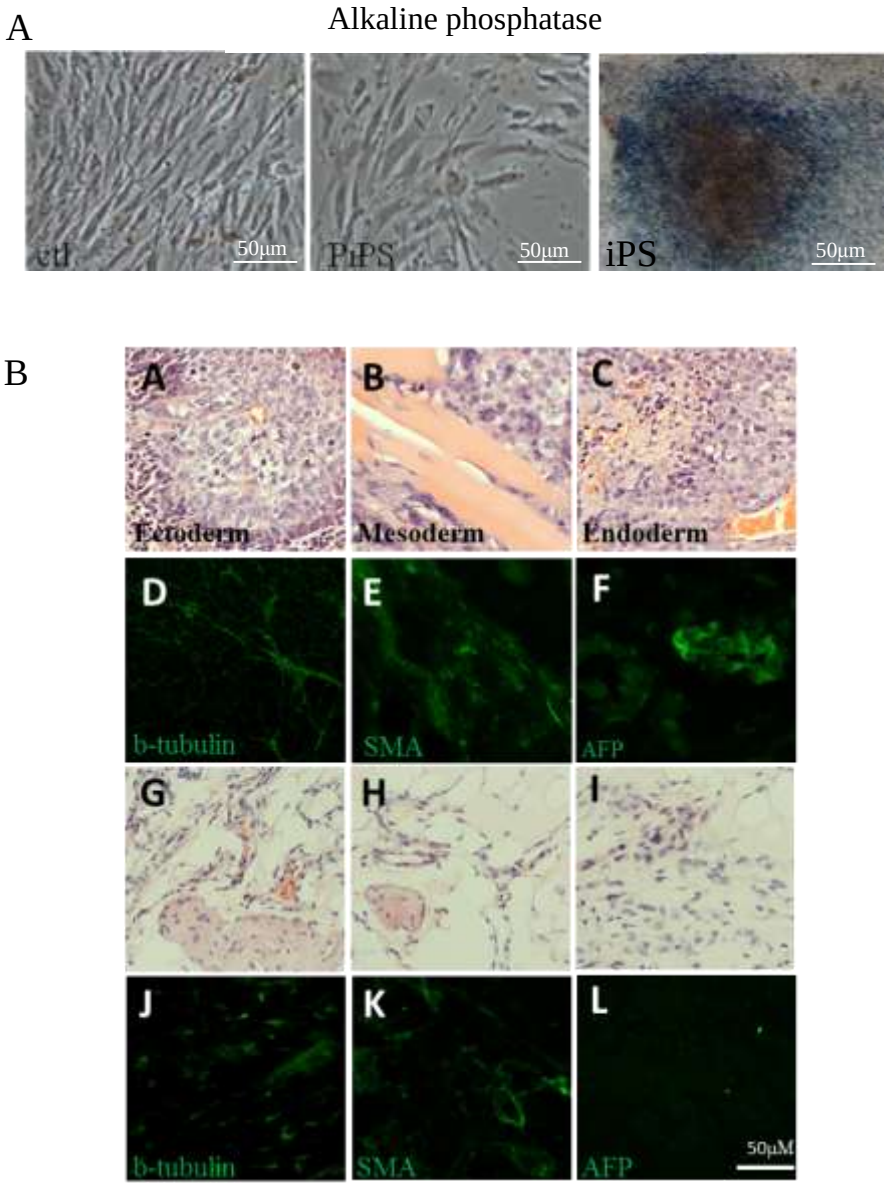


Figure 25. (A) PiPS cells are negative for alkaline phosphatase. Human fibroblasts were reprogrammed for four days and then stained for alkaline phosphatase to assess their pluripotent state. PiPS cells were found to be negative for the aforementioned marker. iPS cells were used as a positive ctl for alkaline phosphatase. **(B) PiPS did not form teratomas *in vivo*.** Subcutaneous transplantation of PiPS cells (G-L) in SCID mice did not result in teratoma formation 2 months after the procedure, unlike iPS (A-F) cell transplantation where all three germ layers were generated as shown by hematoxylin and immunofluorescent staining. β-tubulin III: ectoderm, SMA: mesoderm, AFP: endoderm.

3.3 PiPS cells can differentiate into SMCs

To assess the potential of PiPS cells to differentiate into different lineages in response to specific stimulus and based on observations of expression of VEGFR2 in the aforementioned cells, we questioned whether they could differentiate into vascular lineages and specifically SMCs. Previous studies in our laboratory have demonstrated that Collagen IV induces differentiation of ESCs into SMCs (Xiao et al., 2007). Therefore, we speculated if application of this protocol of differentiation could result in differentiation of PiPS cells into SMCs. In order to test this, PiPS cells were seeded on a Collagen IV substrate and maintained in differentiation medium (DM) for different timepoints. It was revealed that 4 days under the aforementioned conditions were sufficient to change the morphology of PiPS cells into one that resembled the morphology of SMCs as opposed to the control (ctl) population where cells maintained their fibroblast morphology (Figure 26). Differentiation of cells reprogrammed for 2, 4, 7, 14 and 21 days confirmed that 4 days of reprogramming is the optimal time point for obtaining SMC in higher efficiencies (Figures S2, S3). Furthermore, the differentiated PiPS cells expressed SMC specific markers such as SMA and Calponin in a time dependant manner at the mRNA level (Figure 27). More specifically, on day 4 of differentiation PiPS-SMCs expressed a full panel of SMC specific markers at the mRNA level (Figure 28A) and the SMC markers Calponin and SM22 at the protein level (Figure 28B). Interestingly, induction of SMC marker mRNA at day 4 of PiPS cell differentiation was found to be greater than that of 6 day differentiated iPS cells under the same differentiation conditions (Figure S4). Finally, indirect immunofluorescent staining for Calponin and SM22 demonstrated a SMC-like morphology when compared to the ctl cells (Figure 29) with an efficiency of 35.4% for

Calponin and 26.4% for SM22 (Figure S4). These results demonstrate that PiPS cells can differentiate into SMCs and were defined as PiPS-SMCs.

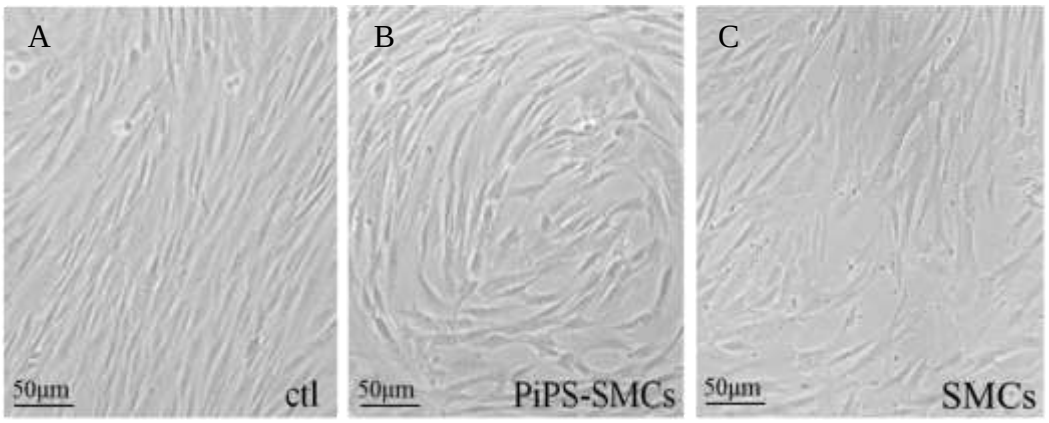


Figure 26. PiPS cells display a SMC-like morphology after 4 days in differentiation conditions. PiPS **(B)** or ctl cells **(A)** were seeded on a Collagen IV substrate and maintained in DM for 4 days. Light microscope images reveal that PiPS-SMCs have adopted a SMC-like morphology as comparable to native SMCs **(C)**. Images were taken with the Nikon Eclipse TS100. (Image representative of at least 3 experiments)

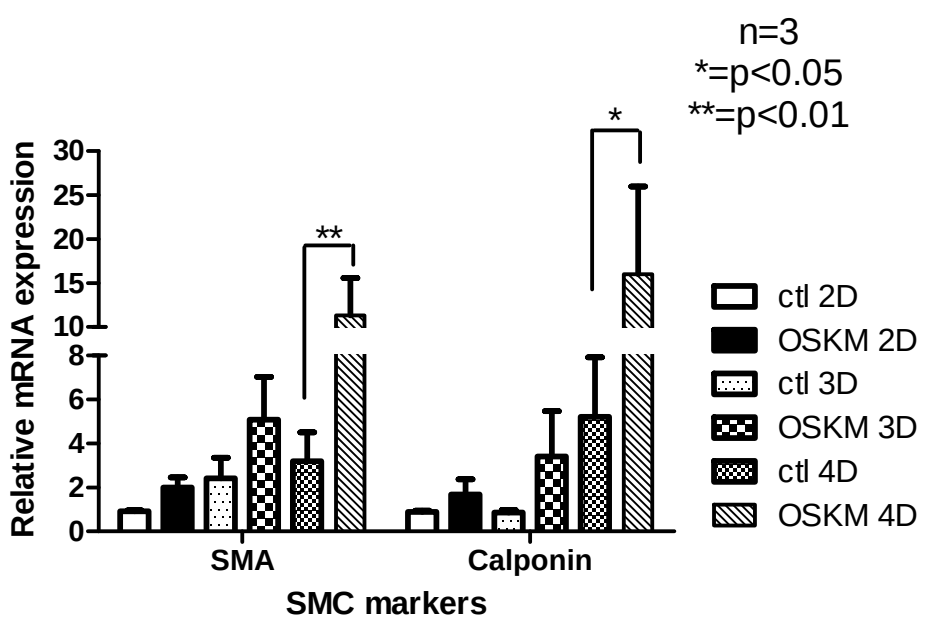


Figure 27. SMC markers are upregulated in a time dependant manner during the differentiation of PiPS cells into SMCs. PiPS or ctl cells were seeded in differentiation conditions and samples were harvested at different timepoints (2, 3 and 4 days). Q-PCR revealed that there is an upregulation of SMC specific markers during PiPS cell differentiation in a time dependant manner. 18s was used as an internal control (mean ±SEM of 3 individual experiments)

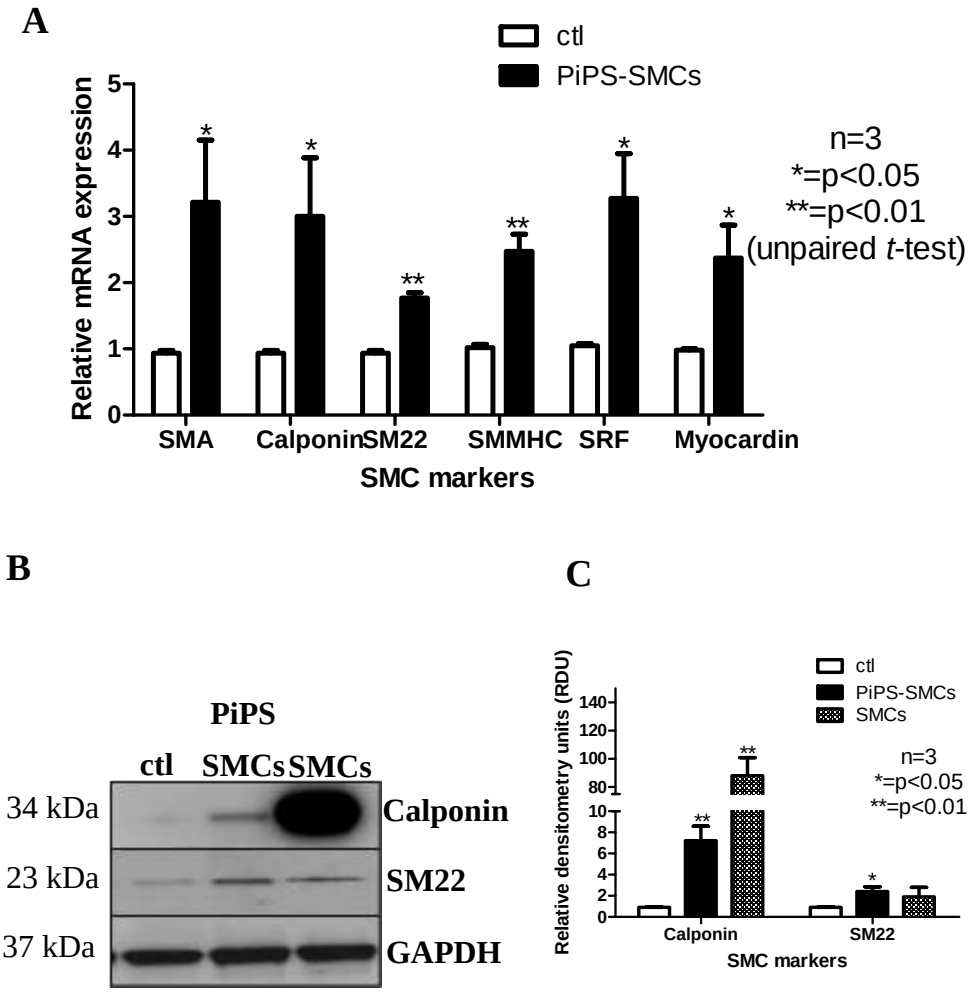


Figure 28. PiPS cells express SMC specific markers after 4 days in differentiation conditions. PiPS cells or ctl cells were differentiated on Collagen IV and maintained in DM medium. Samples were harvested after 4 days and mRNA and total protein were extracted. Q-PCR **(A)** (the mean ±SEM of 3 individual experiments is shown) and western blot analysis **(B)** revealed an upregulation of SMC specific markers in PiPS-SMCs at the mRNA and protein levels respectively, when compared to the ctl cells. SMCs were used as a positive ctl (image representative of 3 experiments). (C) Quantification of SMC marker upregulation at the protein level (the mean ±SEM of 3 individual experiments).

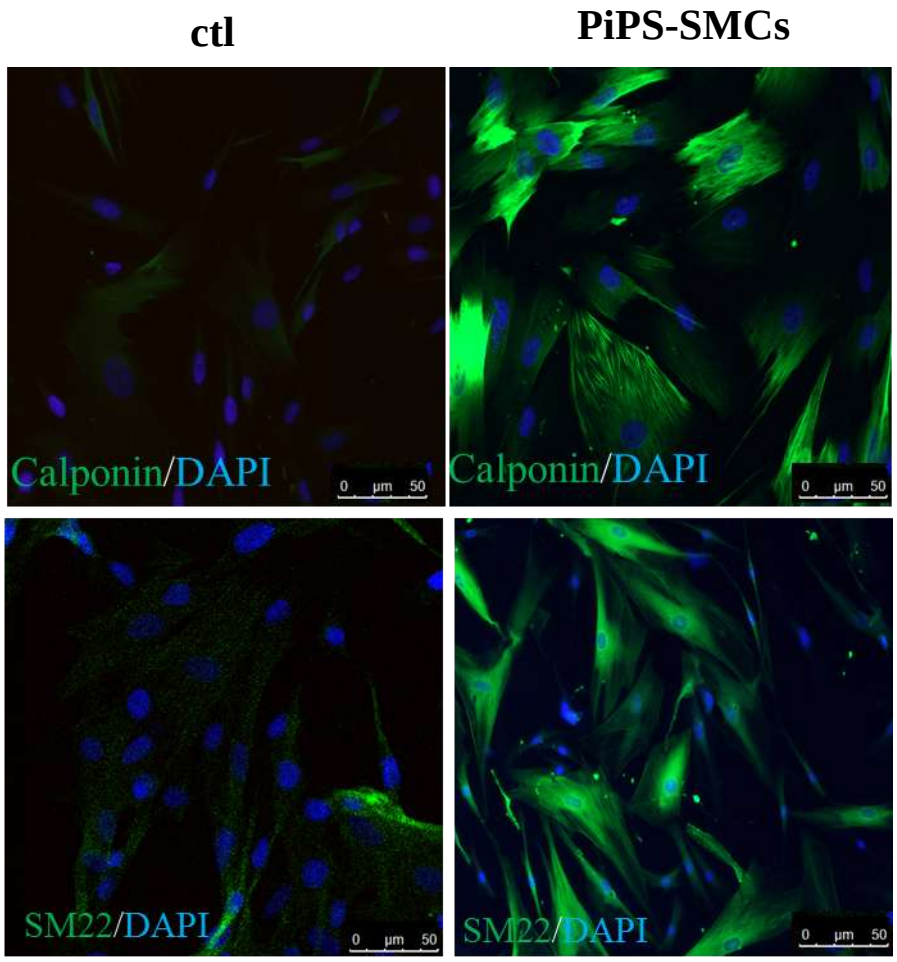


Figure 29. Four days differentiated PiPS cells were positive for Calponin and SM22 and demonstrated a typical SMC-like morphology. PiPS or ctl cells were differentiated for 4 days and then stained for the SMC markers Calponin and SM22. DAPI was used as counterstaining for identification of the nucleus. The cells were positive for the aforementioned SMC markers and exhibited a SMC-like morphology. Images of indirect immunofluorescent staining were captured using SP5 confocal microscopy and were representative of at least 3 experiments.

3.4 DKK3 is involved in the differentiation of PiPS cells into SMCs.

To elucidate the mechanism involved in the PiPS-SMCs differentiation, selected genes identified in the microarray analysis were screened at the mRNA level (Figure 30A). Among the many candidates, a member of the Dickkopf family, DKK3, was selected to be studied further. DKK3 was found to be upregulated in parallel with SMC markers during the PiPS-SMC differentiation at both the mRNA (Figure 30B) and protein levels (Figure 30C). Furthermore, indirect immunofluorescent staining confirmed the concomitant expression of DKK3 with the SMC specific markers Calponin and SM22 and revealed a stronger perinuclear pattern of DKK3 in the differentiated PiPS-SMCs when compared to the ctl cells (Figure 31). To investigate whether DKK3 is involved in PiPS-SMC differentiation, DKK3 was transiently overexpressed via nucleofection in PiPS-SMC during differentiation, for 48 hours. PiPS-SMCs were also nucleofected with a pCMV5 empty vector and were used as a control (ctl). Upregulation of DKK3 lead to further induction of SMC specific markers at both the mRNA (Figure 32A) and protein levels (Figure 32B). Preliminary results show that upregulation of DKK3 in PiPS cells does not increase proliferation. Although total cell count shows a small increase in cell numbers, employment of BrdU assay upon overexpression of DKK3 does not reveal a significant induction of proliferation (Figure S4). These results suggest that DKK3 is involved in PiPS-SMC differentiation. The next crucial point was to investigate whether DKK3 is sufficient to drive the mechanism of PiPS-SMC differentiation. In order to address this question, the aforementioned gene was suppressed via lentiviral delivery of DKK3 shRNA for 48 and 72 hours during differentiation. PiPS-SMCs infected with the non targeting shRNA lentivirus were used as the ctl population. Interestingly, SMC specific markers were found to be downregulated at both the protein (Figure 33A) and mRNA levels (Figure 33B) concomitant to the downregulation of DKK3.

Indirect immunofluorescence also revealed that there is a reduced differentiation potential in PiPS-SMCs after DKK3 silencing for 72 hours (Figure S6).

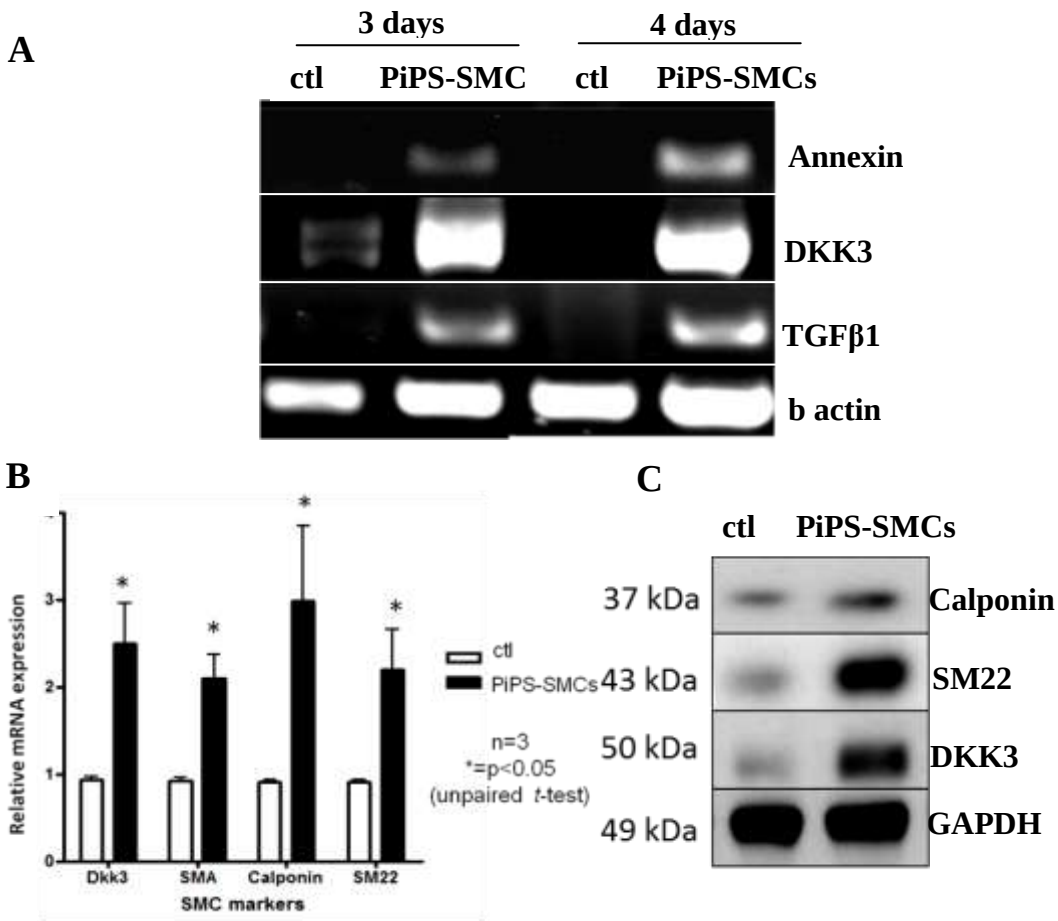


Figure 30. DKK3 is upregulated during differentiation of PiPS-SMCs. (A) Figure representative of conventional PCR investigating the expression of a number of genes identified in the microarray analysis, for their involvement during PiPS-SMCs differentiation. DKK3 was found to be significantly upregulated during 3 and 4 days of PiPS-SMCs differentiation along with Annexin3 and TGFβi. DKK3 expression was upregulated in parallel with SMC specific markers on day 4 of differentiation in PiPS-SMCS at both the mRNA and protein levels as revealed by Q-PCR **(B)** (the mean ±SEM of 3 individual experiments is shown) and western blot analysis **(C)** (image representative of 3 experiments) respectively.

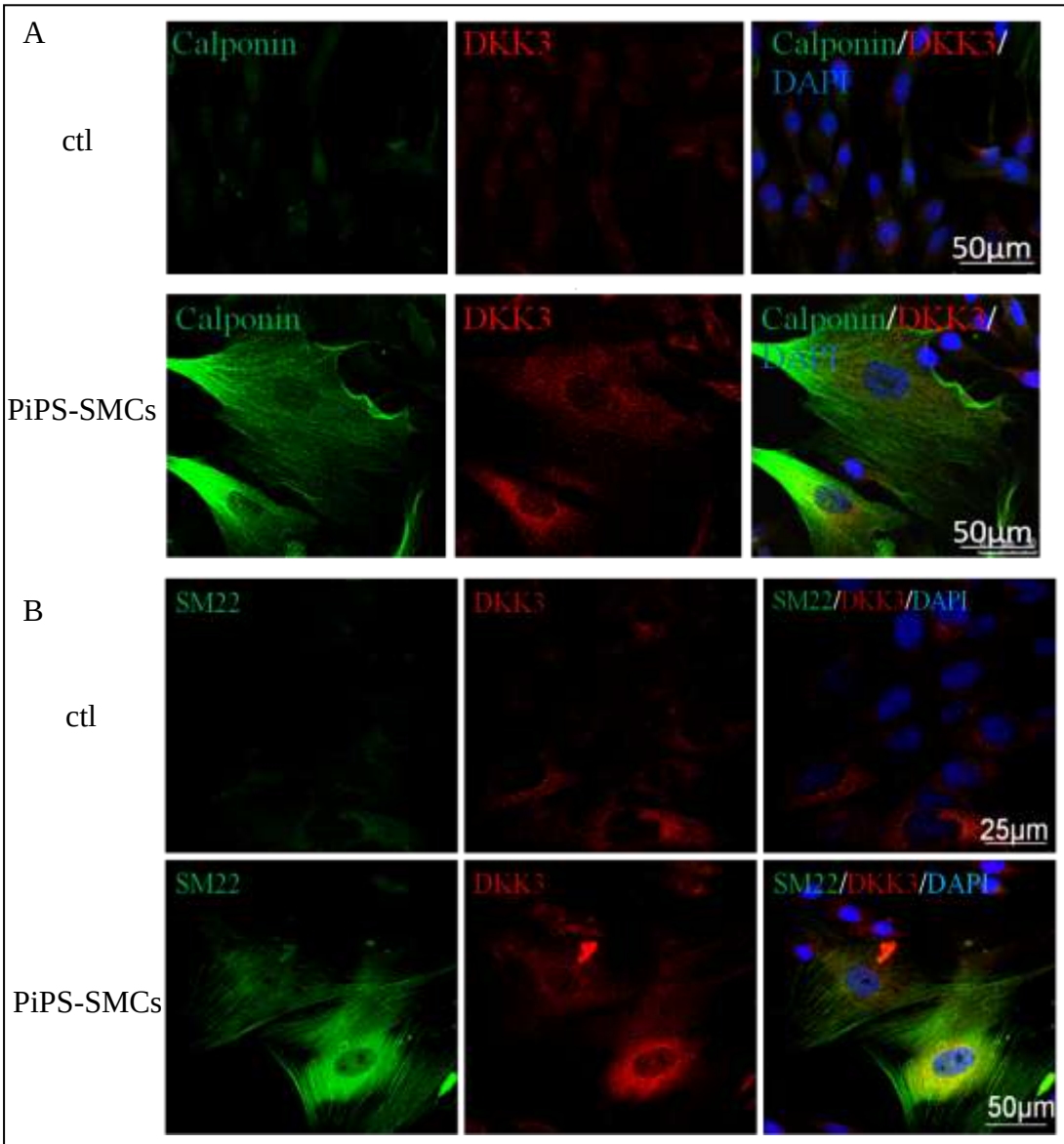


Figure 31. DKK3 is expressed in a perinuclear pattern concomitant with SMC specific markers. Double indirect immunofluorescent staining for **(A)** Calponin-DKK3 and **(B)** SM22-DKK3 revealed a concomitant expression of SMC markers and DKK3 in PiPS-SMCs. PiPS or ctl cells were differentiated for 4 days and then double stained for Calponin-DKK3 and SM22-DKK3. DAPI was used as counterstaining for identification of the nucleus. The staining revealed a perinuclear DKK3 expression concomitant with Calponin and SM22 expression. Images were captured using SP5 confocal microscopy and were representative of 3 experiments.

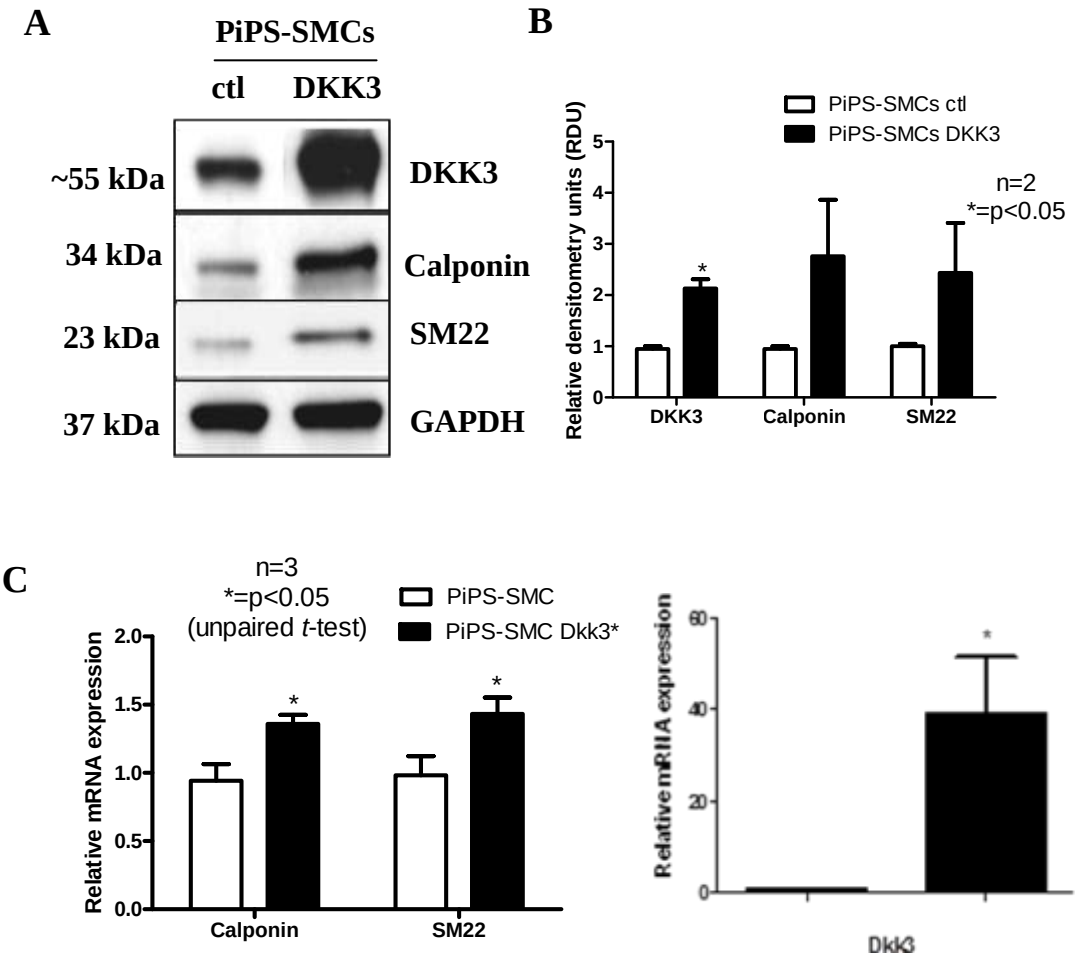


Figure 32. DKK3 overexpresssion further induces the expression of SMC markers in PiPS-SMCs during differentiation. PiPS cells were seeded on a Collagen IV substrate and differentiated in DM for 2 days. They were then nucleofected with DKK3 or a pCMV5 empty vector (2µg/2x10^6 cells) and samples were harvested after 48 hours. **(A)** Western blot analysis (image representative of 2 experiments) accompanied by quantification by densitometry **(C)** as well as Q-PCR **(B)** (the mean ±SEM of 3 individual experiments is shown) revealed a further induction of SMC markers Calponin and SM22 after overexpression of DKK3 on both the protein and mRNA level respectively.

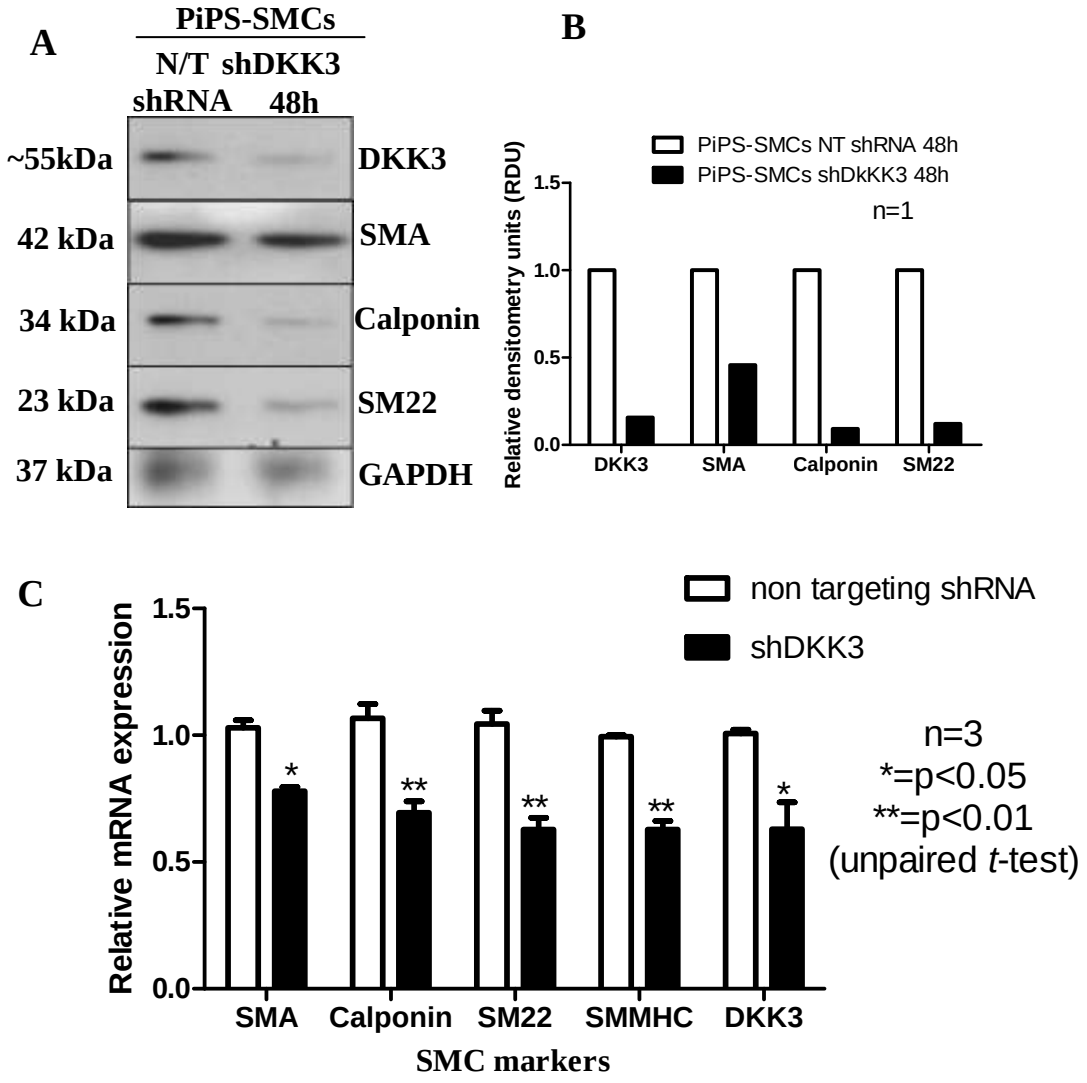


Figure 33. DKK3 downregulation leads to dowregulation of SMC markers in PiPS-SMCs during differentiation. PiPS cells were infected with shDKK3 or non targeting shRNA (10^7TU/ml complemented with 10µg/ml polybrene) 24 hours after being seeded on Collagen IV and DM. Samples were harvested 48 and 72 hours post infection and were subjected to **(A)** western blot analysis for SMC specific markers. **(C)** Quantification of the protein for the 48 hour timepoint revealed downregulation of a panel of SMC specific markers. mRNA was also extracted 72 hours post infection and the relative expression of SMC specific markers was determined on the transcriptional level by **(B)** Q-PCR (the mean ±SEM of 3 individual experiments is shown).

3.5 The cytokine role of DKK3 in PiPS-SMC differentiation

DKK3 is a secreted glycoprotein which is expressed in a variety of tissues and its role as a cytokine has previously been reported (Watanabe et al., 2009). However, its physiological role remains unclear. To investigate the potential role of DKK3 as a cytokine in the differentiation of PiPS-SMC, the supernatant of 4 day differentiated PiPS and ctl cells was collected and then subjected to western blot analysis. The results revealed that there was an increased release of DKK3 glycoprotein in the supernatant of PiPS-SMCs when compared to the supernatant of the ctl cells (Figure 34A and 34B). These findings were further confirmed by DKK3 ELISA measurements, where the concentration of released DKK3 was detected. Quantification of the released DKK3 showed a higher concentration of the glycoprotein in the supernatant of PiPS-SMCs when compared to that of the ctl cells (concentrations as high as 80ng/ml) (Figure 34C).

To assess whether the changes in expression of DKK3 at the protein and mRNA level were linked to its secretion, western blot analysis of the supernatant was also performed in samples where DKK3 was transiently overexpressed in PiPS-SMCs during differentiation. Quantification via DKK3 ELISA revealed that upregulation of DKK3 at the mRNA level lead to extracellular secretion of the glycoprotein. The supernatant of the differentiated PiPS cells which were nucleofected with an empty pCMV5 vector was used as a control (Figure 35A). Furthermore, supernatant of PiPS-SMCs in which DKK3 was suppressed via lentiviral delivery of DKK3 shRNA in PiPS-SMCs during differentiation, was analysed. PiPS-SMCs were also infected with non targeting shRNA, the supernatant of which was used as a control. Western blot analysis of the aforementioned samples revealed that there is a decreased release of the glycoprotein in the supernatant after successful downregulation at the mRNA (Figure 35B).

Finally in order to ascertain whether DKK3 has a cytokine effect in our system of differentiation, a human recombinant DKK3 cytokine was utilized in order to stimulate PiPS-SMCs. PiPS cells were seeded on a Collagen IV substrate and maintained in DM for 3 days. They were then stimulated with 50ng/ml of human recombinant DKK3 for 24 hours. The activity of human recombinant DKK3 protein is confirmed by western blot analysis in Figure S7. Unstimulated PiPS-SMCs cells served as the control. Analysis of the samples harvested 24 hours following stimulation, revealed an upregulation of SMC specific marker expression at both the mRNA (Figure 36A) and protein (Figure 36B) levels between stimulated and unstimulated PiPS-SMCs, as shown by Q-PCR and western blot analysis respectively.

These results suggest that DKK3 secretion plays an important role in PiPS-SMC differentiation and that stimulation of PiPS-SMCs with recombinant DKK3 leads to further induction of SMC markers in our SMC differentiation system.

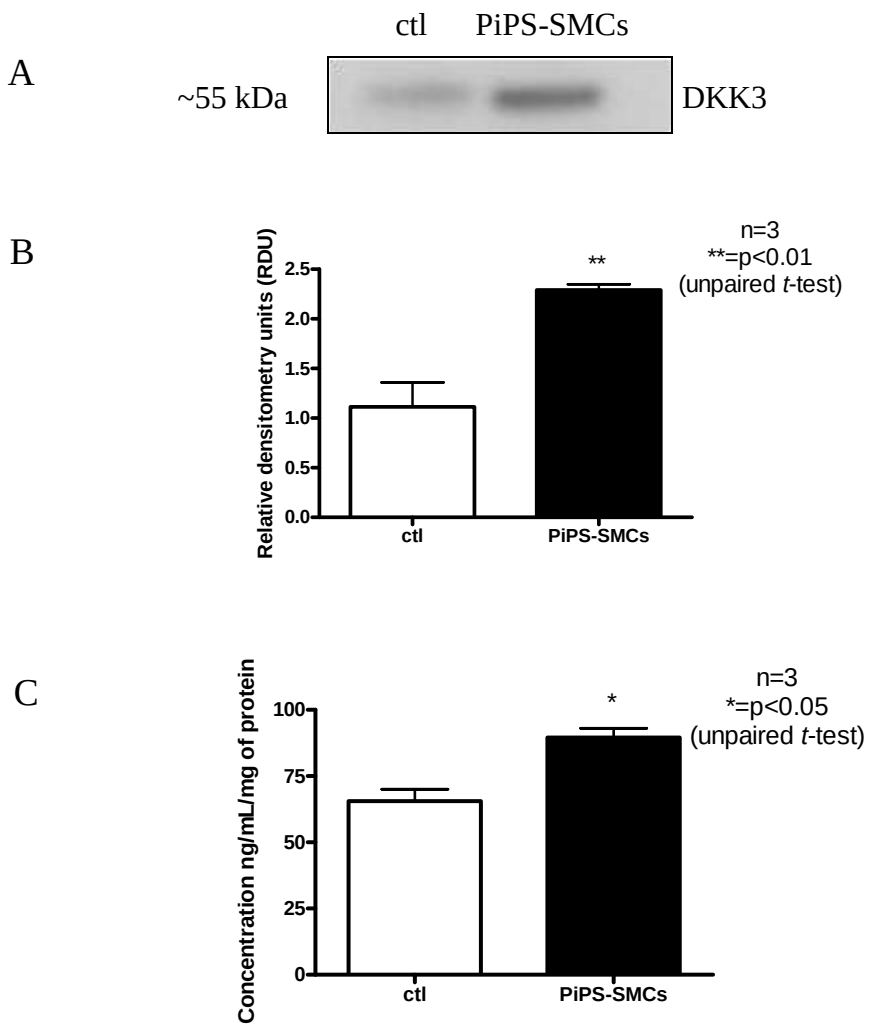
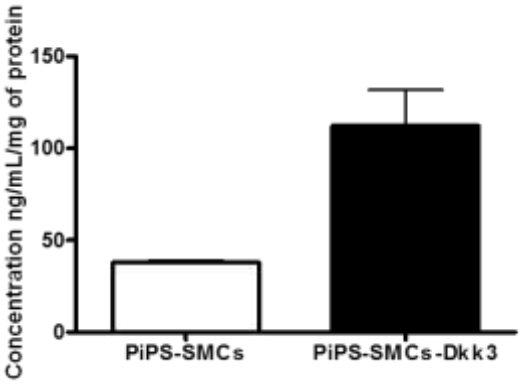


Figure 34. Secretion of DKK3 is increased during differentiation of PiPS into SMCs. PiPS and ctl cells were differentiated on Collagen IV and maintained in DM for 4 days. The supernatant from both populations was collected and 20µl of each sample were subjected to western blot analysis as described in paragraph 2.2.15. **(A)** Immunoblotting for DKK3 revealed an increased release of DKK3 in the differentiated PiPS-SMCs when compared to the ctl cells. **(B)** Densitometry of 3 independent experiments (the mean ±SEM of 3 individual experiments is shown). **(C)** Quantification of DKK3 concentartion in the supernatant of both populations by ELISA, reveals that there is a higher concentration of DKK3 glycoprotein in the supernatant of PiPS-SMCs when compared to the supernatant of the ctl cells.

A



n=2

p=0.06

B

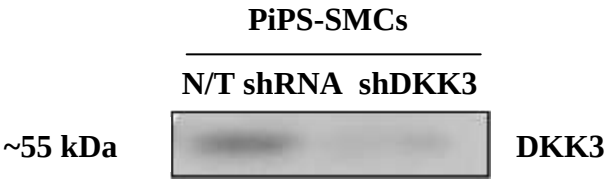


Figure 35. Transcriptional upregulation or downregulation of DKK3 is related to its secretion to the supernatant. (A) PiPS cells were differentiated for 2 days and were then forced to overexpress DKK3 or an empty pCMV5 vector (2μg/2x10^6 cells) via nucleofection for an additional 2 days. Supernatants from both samples were collected and the concentraton of DKK3 was detected by DKK3 ELISA. Quantification of DKK3 in the supernatant revealed an increased release of the glycoprotein after forced expression of DKK3 at the mRNA level. **(B)** PiPS cells were seeded on Collagen IV and 24 hours later were infected with DKK3 shRNA or non targeting shRNA (10^7TU/ml complemented with 10μg/ml polybrene). The supernatants were collected 72 hours later and 20μl of each sample were subjected to western blot analysis as described in paragraph 2.2.15. Silencing of DKK3 via lentiviral delivery of DKK3 shRNA lead to decreased release of the glycoprotein in the supernatant (image representative of 2 experiments).

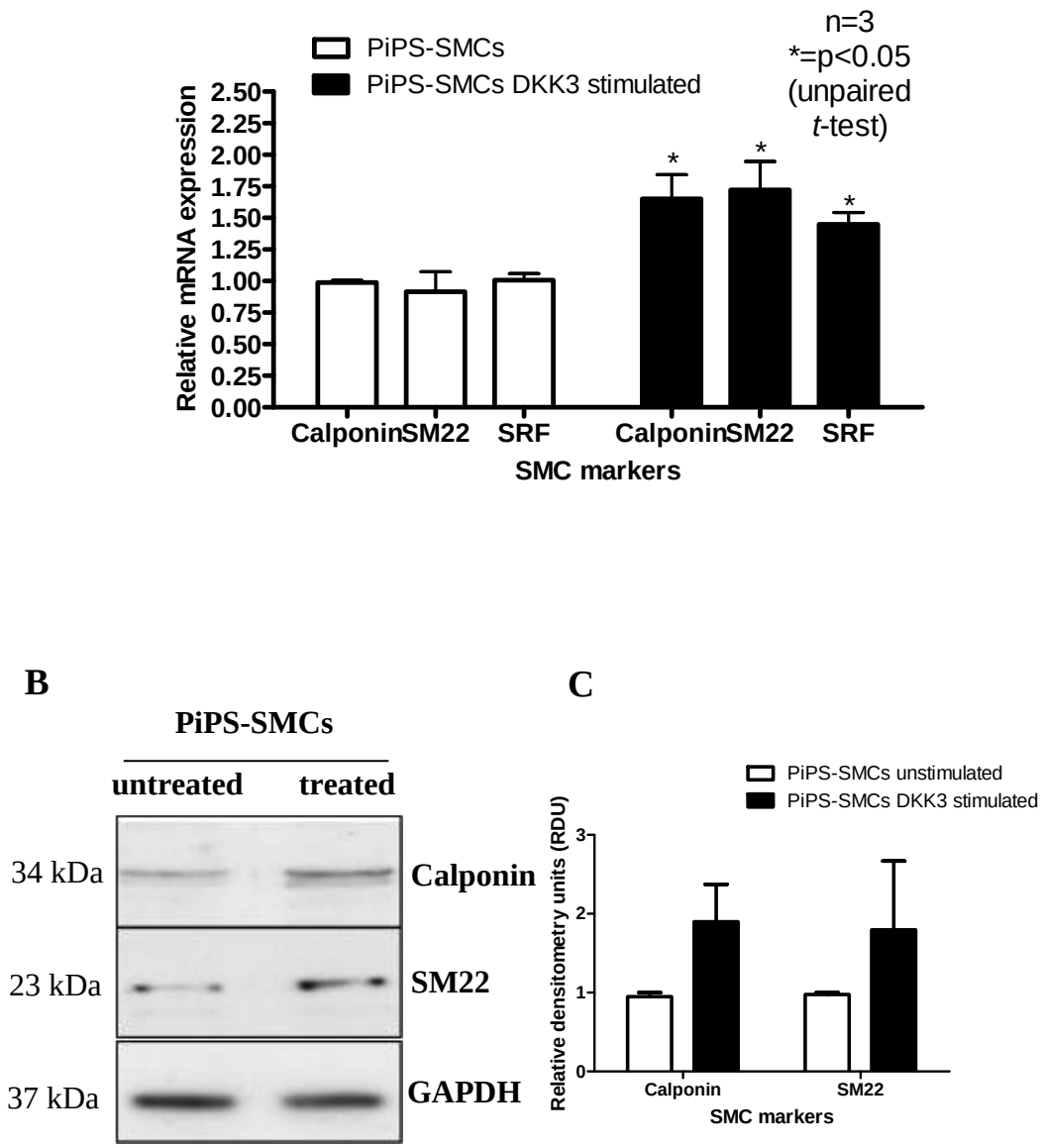


Figure 36. DKK3 stimulation leads to upregulation of SMC markers. PiPS cells were differentiatied on Collagen IV and DM. They were then stimulated with 50ng/ml human recombinant DKK3 cytokine for 24 hours. Treated and untreated samples were harvested and RNA and protein were extracted. Q-PCR **(A)** and western blot analysis **(B)** after densitometry **(C)** revealed that there is a further induction of SMC markers such as Calponin and SM22 at the mRNA and protein levels respectively (the mean ±SEM of 3 individual Q-PCR experiments is shown).

3.6 DKK3 regulates the transcriptional activation of SM22

Thus far, we have shown that DKK3 overexpression and stimulation lead to upregulation of SMC markers at both the transcriptional and protein level. In order to shed light into the molecular mechanism regulated by DKK3 during PiPS-SMCs differentiation, the effect of DKK3 in the transcriptional activation of SMC specific markers was evaluated. Luciferase assays were performed and the promoter activity of SM22 was detected. PiPS cells were seeded on Collagen IV for 24 hours in order to initiate differentiation. The cells were then transfected with a reporter gene PGL3-Luc-SM22 vector (Margariti et al., 2009) (0.33μg/well) together with the DKK3 plasmid in a dose response manner (0.8/0.16/0.25μg/well). A PGL3-Renilla vector (0.1μg/well) was included in all transfection assays as the internal control. Simultaneously, cells were transfected with an empty pCMV5 vector (0.16μg/well), a PGL3-Luc-SM22 vector (0.33μg/well) and PGL3-Renilla (0.1μg/well) and comprised the control population. The samples were harvested 48 hours later and the Luciferase and Renilla enzymatic activities were detected (Promega Luciferase assay). Analysis of the results revealed that there was an upregulation of the SM22 promoter activity upon DKK3 overexpression suggesting that DKK3 induced activation of SM22 at the transcriptional level (Figure 37).

To further confirm the effect of DKK3 in the transcriptional activation of SM22, the promoter activity of SM22 was also measured after DKK3 silencing. PiPS cells were seeded on Collagen IV for 24 hours to initiate differentiation after which DKK3 was suppressed by lentiviral delivery of DKK3 shRNA. Simultaneously, 24 hour differentiated PiPS cells were infected with non targeting shRNA and comprised the control population. 24 hours after the infection both populations were co transfected

with PGL3-Luc-SM22 (0.33μg/well) and PGL3-Renilla vectors (0.1μg/well).

The samples were harvested 48 hours post transfection and the luciferase enzymatic activity was measured. The renilla enzymatic activity was used as an internal control. Interestingly, the results revealed that downregulation of DKK3 in the differentiated PiPS cells lead to downregulation of the SM22 promoter activity, confirming the effect of DKK3 in the transcriptional activation of SM22 (Figure 38).

Finally in order to further evaluate the response of SM22 after DKK3 stimulus, indirect immunofluorescent staining was performed during different timepoints of stimulation with human recombinant DKK3. PiPS-SMCs were stimulated with 50ng/ml of human recombinant DKK3 cytokine and were then stained for both DKK3 and SM22. Surprisingly, it was found tha 4 hours are sufficient to further induce SM22 expression in PiPS-SMCs as revealed by SM22 staining (Figure 39A) and estimation of differentiation efficiency after cell counting (Figure 39B).

The above findings show that DKK3 induces SMC differentiation of PiPS cells by inducing the expression of the SMC specific marker SM22.

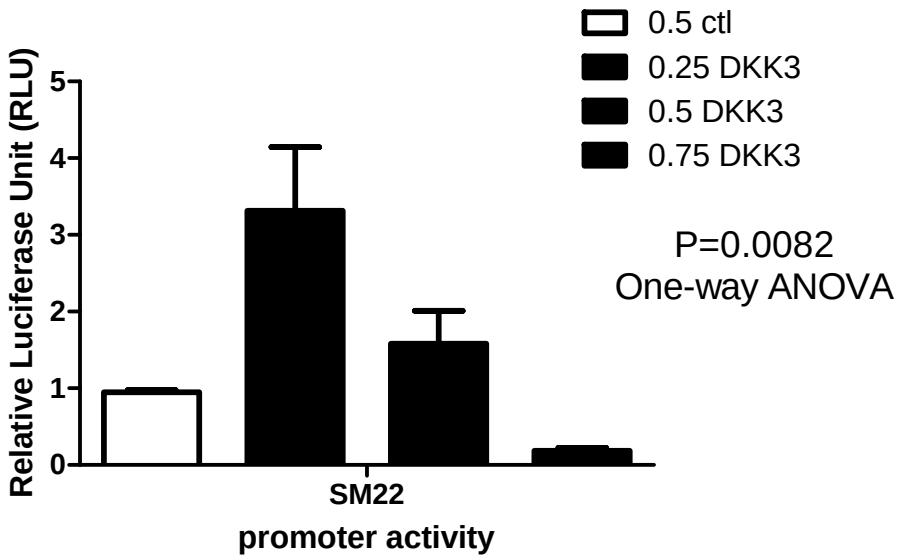


Figure 37. DKK3 overexpression leads to promoter activation of SM22. PiPS cells were seeded on Collagen IV for differentiation and 24 hours later they were co transfected with different concentrations of DKK3 (0.08/0.16/0.25 μg/well) or an empty pCMV5 vector (0.16μg/well), a PGL3-Luc-SM22 vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well). The samples were harvested 48 hours later and their luciferase enzymatic activity was measured with the Lumat LB 9507 Luminometer. The readings showed that there was an increased SM22 promoter activation in the samples overexpressing DKK3 at concentrations 0.08 and 0.16 μg/well when compared to the control samples. The Renilla readings were used as an internal control (the mean ±SEM of 3 individual experiments is shown) (Relative luciferase unit (RLU) was defined as the ratio of luciferase activity to Renilla activity with that of control set as 1.0).

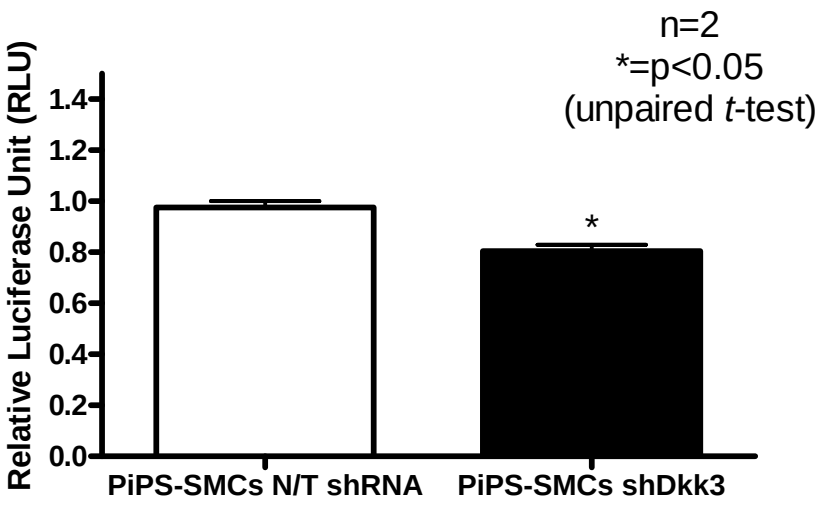
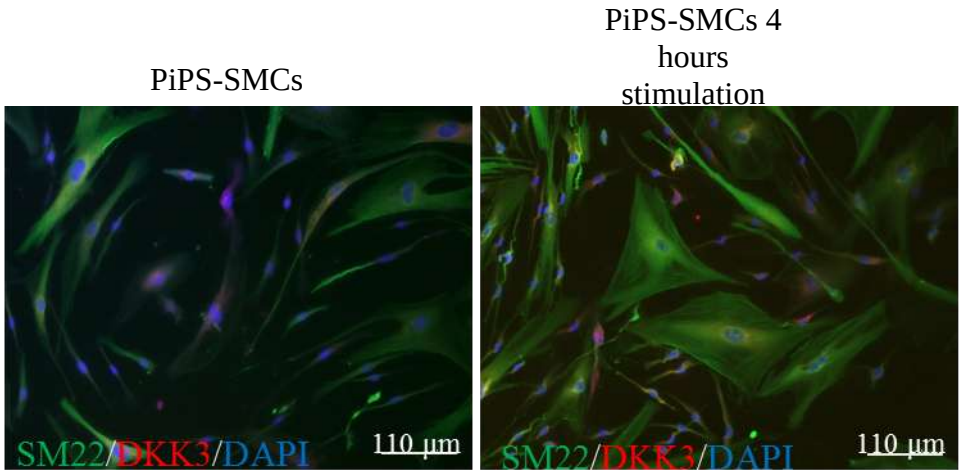


Figure 38. DKK3 silencing leads to downregulation of the SM22 promoter activity. PiPS cells were seeded on Collagen IV and 24 hours later were infected with DKK3 shRNA or non targeting shRNA(10^7 TU/ml complemented with 10μg/ml polybrene). 24 hours after the infection the differentiated PiPS cells were co transfected with a PGL3-Luc-SM22 vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well). Both samples were harvested 48 hours after transfection and the luciferase enzymatic activity was measured with the Lumat LB 9507 Luminometer. Renilla readings were used as an internal control. The results revealed that DKK3 silencing leads to downregulation of the SM22 promoter activity suggesting that DKK3 directly regulates the transcriptional patterns of SM22 (the mean ±SEM of 2 individual experiments is shown) (Relative luciferase unit (RLU) was defined as the ratio of luciferase activity to Renilla activity with that of control set as 1.0).

A



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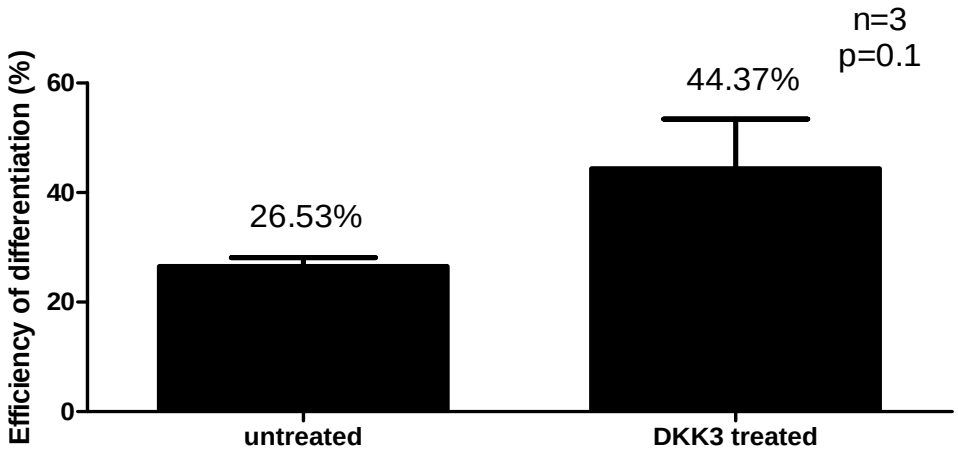


Figure 39. DKK3 stimulation leads to increased expression of SM22 from as early as 4 hours. (A) PiPS-SMCs were stimulated with 50ng/ml human recombinant cytokine for 4 hours and then double stained for SM22 and DKK3 while DAPI was used as counterstaining to identify the nucleus. Images were captured using confocal SP5 microscopy. **(B) Cell counting for SM22 positive cells.** The efficiency of differentiation was calculated as the ratio of SM22 positive cells to the total number of cells field of view (n=3)

3.7 DKK3 activates Wnt signalling during differentiation of PiPS cells into SMCs.

It has previously been shown that β catenin, the downstream mediator of Wnt signalling, directly regulates the transcription of SM22 by binding to its −213 to −192 upstream promoter region (Shafer and Towler, 2009). Furthermore, DKK3 as part of the Dickkopf family of Wnt inhibitors has been implicated in activation or deactivation of Wnt signalling. Its role however remains unclear (Nakamura et al., 2007; Yue et al., 2008). According to our findings, DKK3 regulates the transcriptional activation of SM22. Therefore, in order to test if DKK3-induced PiPS cell differentiation to SMCs occurs through interaction of DKK3 with the Wnt signalling pathway, the activation status of Wnt was initially assessed. A TopFlash vector (TCF reporter plasmid) was utilised to measure the promoter activity of the target genes of Wnt signalling. PiPS cells were seeded on Collagen IV and 24 hours later were co transfected with DKK3 (0.08/0.16/0.25μg/well), the TopFlash vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well). At the same time, 24 hour differentiated PiPS were co transfected with an empty pCMV5 vector (0.16μg/well), the TopFlash vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well) which comprised the control population. The samples were harvested 48 hours later and the luciferase enzymatic activity was measured. The reading for the Renilla enzymatic activity was used as an internal control. Analysis of the results revealed that there is an upregulation in the enzymatic activity of the TopFlash reporter genes after overexpression of DKK3 (Figure 40). These findings suggest that upregulation of DKK3 leads to activation of Wnt signalling during the differentiation of PiPS cells into SMCs.

To confirm the activation of Wnt signalling during PiPS-SMCs differentiation, the transcriptional patterns of the Wnt signalling mediator β catenin was assessed during PiPS cell differentiation to SMCs. PiPS cells were differentiated on Collagen IV for 4 days, mRNA was extracted and the samples were subjected to Q-PCR. Analysis of the results revealed that there was an upregulation of the expression of β catenin in PiPS-SMCs concomitant to the upregulation of DKK3 (Figure 41).

To further investigate the Wnt signalling activation status in PiPS-SMCs and the involvement of DKK3 in that process, the transcriptional patterns of additional targets of the aforementioned signalling pathway were assessed after DKK3 overexpression. DKK3 or an empty pCMV5 vector (2μg/2x10^6 cells) were transiently overexpressed in PiPS-SMCs via nucleofection and mRNA and protein were extracted from both samples. Q-PCR revealed that there is an upregulation in the expression of the Wnt signalling targets β catenin, TCF1 and Axin2 at the transcriptional level after forced expression of DKK3 in PiPS-SMCs (Figure 42). Furthermore, the transcriptional levels of the aforementioned genes were assessed after DKK3 silencing in PiPS-SMCs. PiPS-SMCs were viraly infected with DKK3 shRNA or non targeting shRNA and samples were harvested 72 hours later. mRNA and protein were harvested and samples were subjected to Q-PCR and western blot analysis respectively. Interstingly it was found that β catenin, TCF1 and Axin2 were downregulated at the transcriptional level after downregulation of DKK3 in PiPS-SMCs (Figure 43A). Finally, β catenin was also found to be downregulated at the protein level after DKK3 downregulation (Figure 43B). These results interestingly show that DKK3 can regulate Wnt signalling during PiPS-SMC differentiation and its downstream components.

While trying to further investigate the upregulation of β catenin, the penultimate and key downstream component of Wnt signalling, the expression levels of the upstream Wnt component, GSK3, were assessed. It has been shown that when Wnt signalling is not activated, GSK3 as part of the axin/GSK-3/APC complex promotes the proteolitic degredation of β catenin. Downregulation of GSK3 leads to inhibition of β catenin degradation and therefore its accumulation into the cell and ultimately its translocation to the nucleus (Miller et al., 1999).

The expression of GSK-3 levels were therefore identified in PiPS-SMCs. After 4 days of differentiation, both isoforms of GSK-3, GSK-3a and GSK-3b, were found to be downregulated in PiPS-SMCs when compared to the control cells (Figure 44). These results reinforce our previous findings of Wnt signalling activation during PiPS-SMC differentiation.

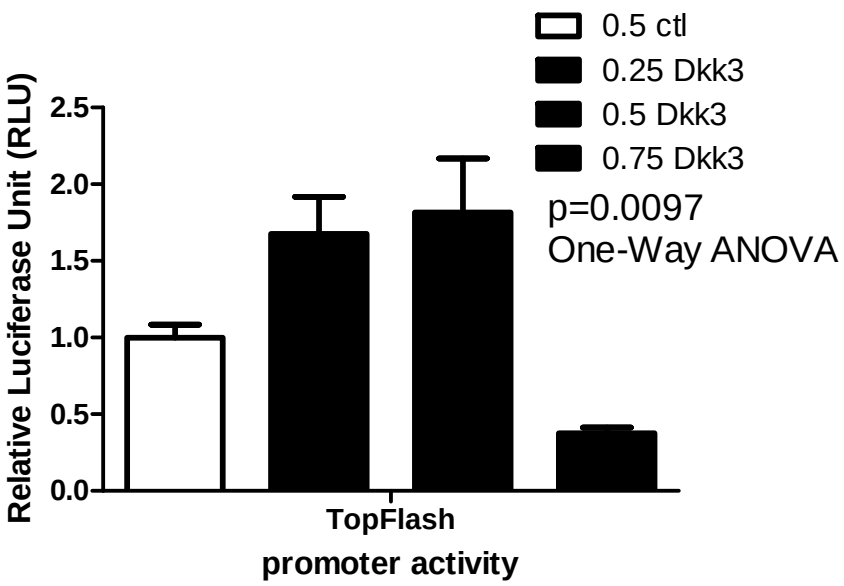


Figure 40. DKK3 activates Wnt signalling during PiPS-SMC differentiation. PiPS cells were seeded on Collagen IV for differentiation and 24 hours later they were co transfected with different concentrations of DKK3 (0.08/0.16/0.25 μg/well) or an empty pCMV5 vector (0.16μg/well), a TopFlash vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well). The samples were harvested 48 hours later and their luciferase enzymatic activity was measured with the Lumat LB 9507 Luminometer. The readings showed that there is an upregulation in the enzymatic activity of the TopFlash reporter genes in the cells overexpressing DKK3 at a concentration of 0.08 μg/well when compared to the control cells. The Renilla readings were used as an internal control (the mean ±SEM of 3 individual experiments is shown) (Relative luciferase unit (RLU) was defined as the ratio of luciferase activity to Renilla activity with that of control set as 1.0).

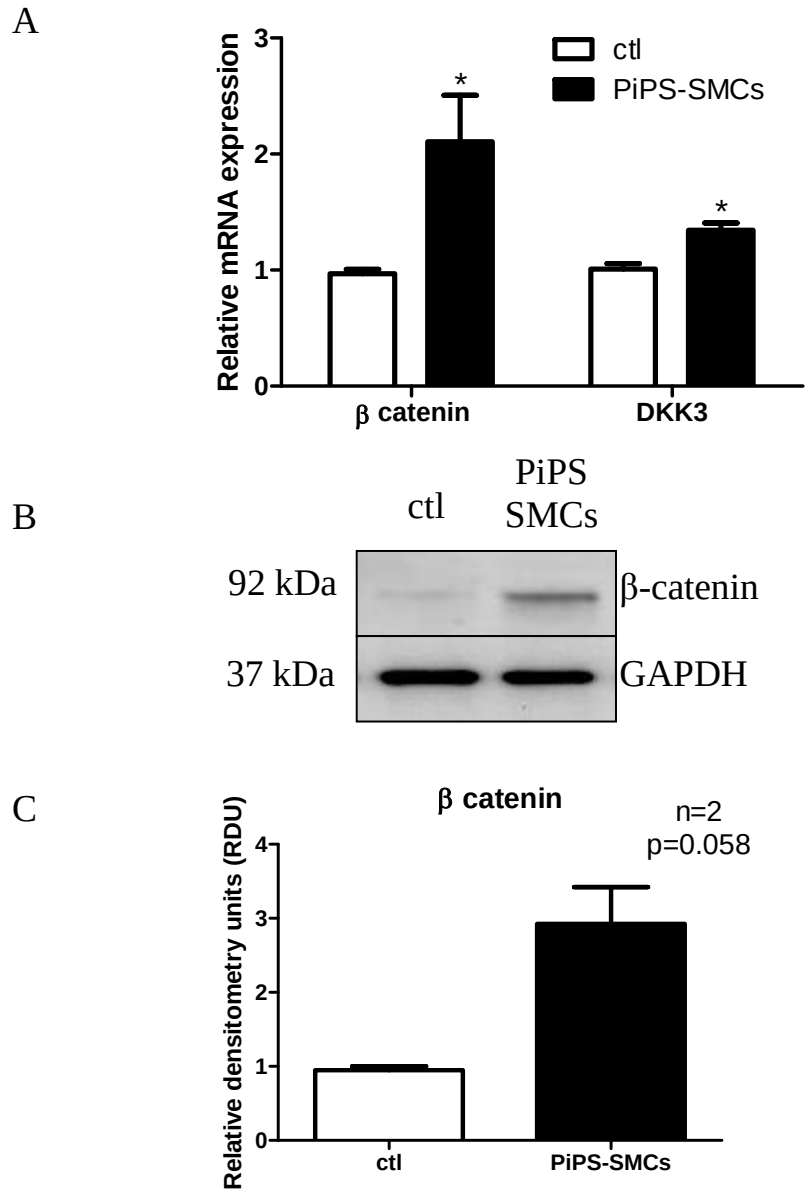


Figure 41. β catenin is upregulated in PiPS-SMCs in parallel with DKK3. PiPS or ctl cells were differentiated on Collagen IV for 4 days after which mRNA and protein were extracted. (A) Q-PCR revealed that there was an upregulation of β catenin in PiPS-SMCs concomitant to the upregulation of DKK3 (the mean ±SEM of 3 individual experiments is shown). Western blot analysis **(B)** and quantification of the protein by densitometry (the mean ±SEM of 2 individual experiments is shown) **(C)** also confirmed upregulation of the increase of intracellular β-catenin.

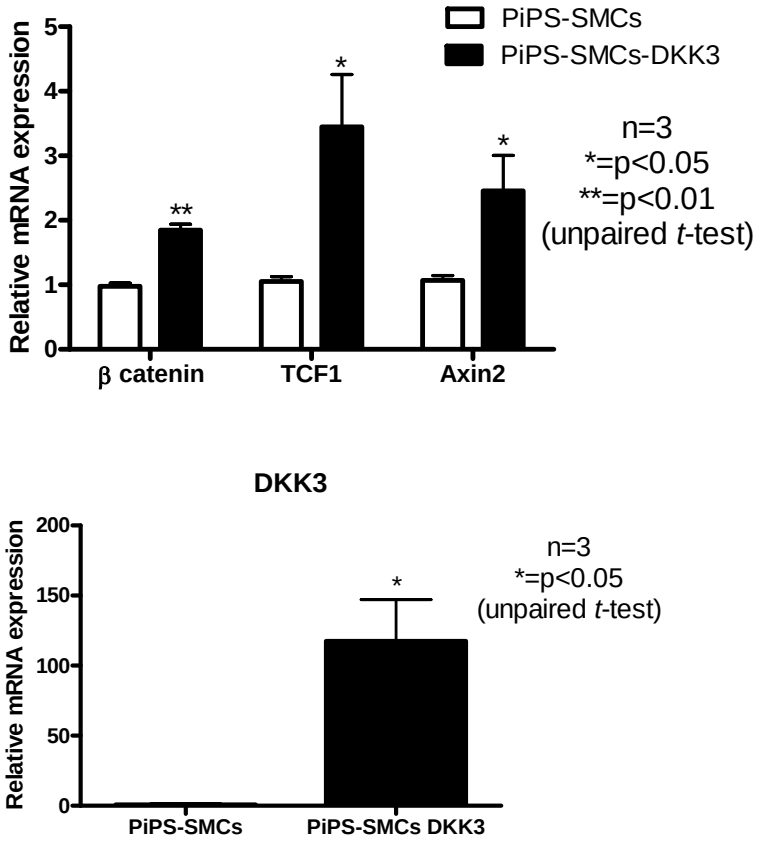


Figure 42. DKK3 overexpression leads to transcriptional upregulation of Wnt signalling target genes. DKK3 or an empty pCMV5 vector (2µg/2x10^6 cells) were transiently overexpressed in PiPS-SMCs via nucleofection and the samples were harvested 48 later. Q-PCR revealed that there is an upregulation of Wnt signalling target genes such as β catenin, TCF1 and Axin 2 at the transcriptional level concomitant with the upregulation of DKK3 (the mean ±SEM of 3 individual experiments is shown).

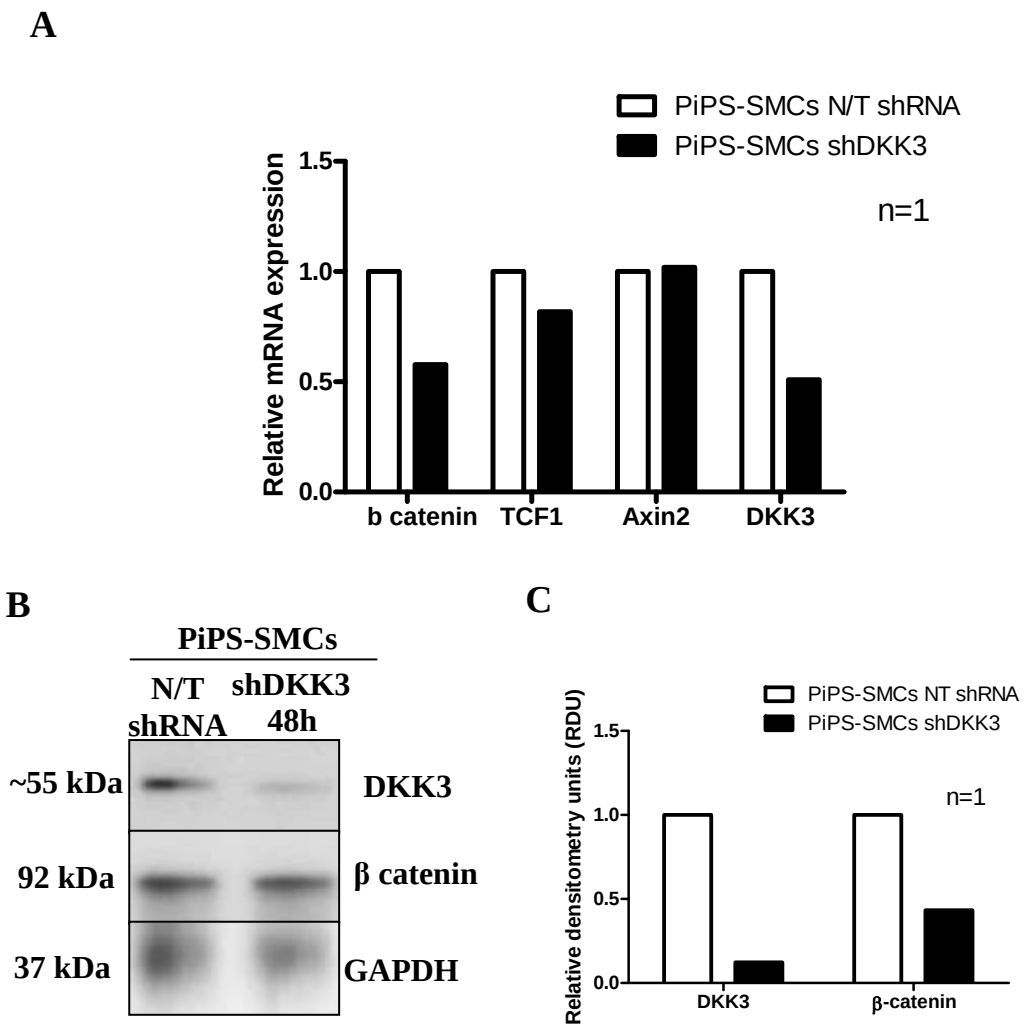


Figure 43. β catenin is downregulated after DKK3 silencing. PiPS cells were seeded on Collagen IV for differentiation and 24 hours later were infected with DKK3 shRNA or non targeting shRNA (10^7TU/ml complemented with 10μg/ml polybrene). The samples were harvested 72 hours later and mRNA and protein were extracted for Q-PCR and wetsrn blot analysis respectively. **(A)** Q-PCR revealed that there is a downregulation of the Wnt signalling targets β catenin, TCF1 and Axin 2 after DKK3 silencing in PiPS-SMCs at the transcriptional level and **(B)** western blot analysis and quantification of the protein by densitometry **(C)** (n=1) confirmed the downregulation of β catenin after DKK3 silencing at the protein level.

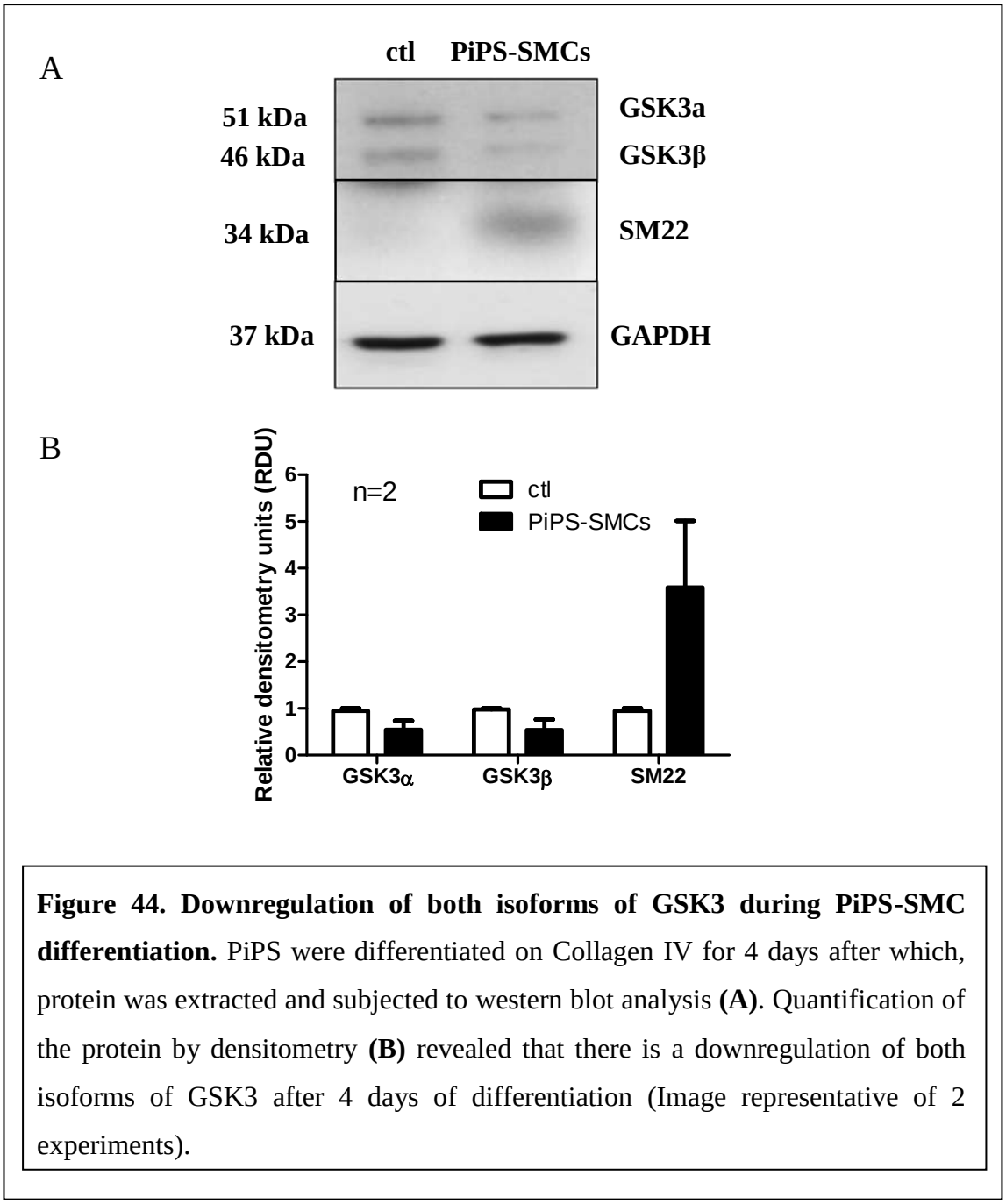


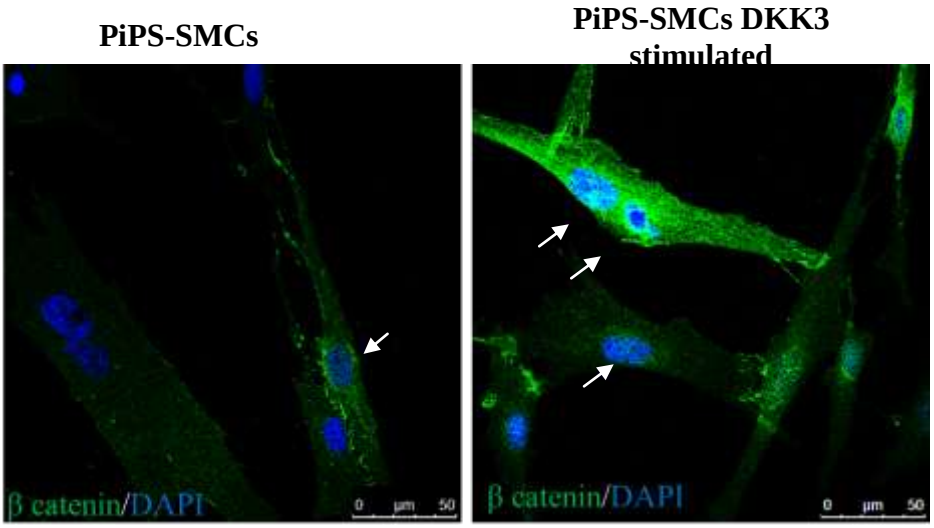
Figure 44. Downregulation of both isoforms of GSK3 during PiPS-SMC differentiation. PiPS were differentiated on Collagen IV for 4 days after which, protein was extracted and subjected to western blot analysis **(A)**. Quantification of the protein by densitometry **(B)** revealed that there is a downregulation of both isoforms of GSK3 after 4 days of differentiation (Image representative of 2 experiments).

3.8 DKK3 induced PiPS-SMC differentiation through β catenin

It has previously been shown that after the inhibition of the β catenin destruction complex, the cytoplasmic β catenin is stabilized and can enter the nucleus in order to interact with the TCF/ LEF family of transcription factors, promoting specific gene expression (Novak and Dedhar, 1999). According to our findings DKK3 forced expression can lead to transcriptional activation of SM22 as well as β catenin and its downstream targets. Therefore, we postulated if DKK3 could induce the translocation of β catenin into the nucleus and subsequently the transcriptional regulation of SM22 and the Wnt signalling target genes. Thus, we examined the localization of β catenin in untreated PiPS-SMCs and PiPS-SMCs upon DKK3 stimulation. Since human recombinant DKK3 is received by the cells from as early as 1 hour (Figure S6), PiPS cells were seeded on Collagen IV for differentiation and 3 days later were stimulated with 50ng/ml of human recombinant DKK3 for up to 6 hours. The samples were then stained for β catenin and DKK3. Indirect immunofluorescent staining revealed that while β catenin appears to be localized in mainly in the cell junctions in PiPS-SMCs, in the cells stimulated with the DKK3 cytokine for 4-6 hours, β catenin has translocated to the nucleus (Figure 45). These results combined with our previous findings suggest that DKK3 may regulate the transcriptional activation of SM22 though activation of Wnt signalling and translocation of its key mediator β catenin to the nucleus. Furthermore, the translocation of β catenin to the nucleus is not a permanent event but instead a dynamic response to DKK3 stimulus for the transcriptional activation of target genes.

In order to confirm that the DKK3 induced regulation of SM22 is mediated by β catenin the reporter activity of SM22 was assessed after DKK3 overexpression with simultaneous suppression of β catenin by shRNA. PiPS cells were seeded on Collagen IV for differentiation and 24 hours later they were infected with β catenin shRNA or non targeting shRNA. 24 hours after the infection the cells were transiently transfected with DKK3 (0.16μg/well), a PGL3-Luc-SM22 vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well) and were maintained in DM for 48 hours. The samples were then harvested and their luciferase enzymatic activity was measured. Interestingly, analysis of the results revealed that silencing of β catenin not only abolished the induction of the transcriptional activity of SM22 after DKK3 overexpression but also causes downregulation of the SM22 promoter activity when compared to the control samples (Figure 46). These results support our previous findings and confirm that the transcriptional activation of SM22 by DKK3 is mediated by β catenin.

A



B

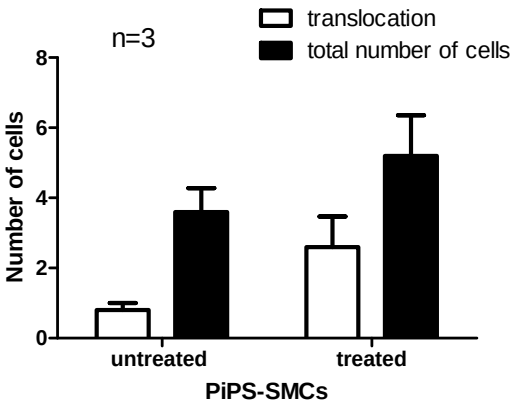


Figure 45. DKK3 stimulation induces β catenin translocation. PiPS cells were differentiated on Collagen IV for 3 days and then stimulated with 50ng/ml human recombinant DKK3 for 6 hours. The cells were then stained for β catenin while DAPI was used as counterstaining to identify the nucleus. **(A)** Indirect immunofluorescent staining revealed that while in the untreated PiPS-SMCs β catenin remains mainly in the cell junctions, in the stimulated cells, β catenin has translocated to the nucleus. (B) Quantification of cells where β catenin has been translocated to the nucleus. In the unstimulated cells, β catenin was translocated to the nucleus whereas in the stimulated cells 13 out of 26 were positive for the aforementioned translocation. Images were captured using SP5 confocal microscopy (Image representative of 3 experiments).

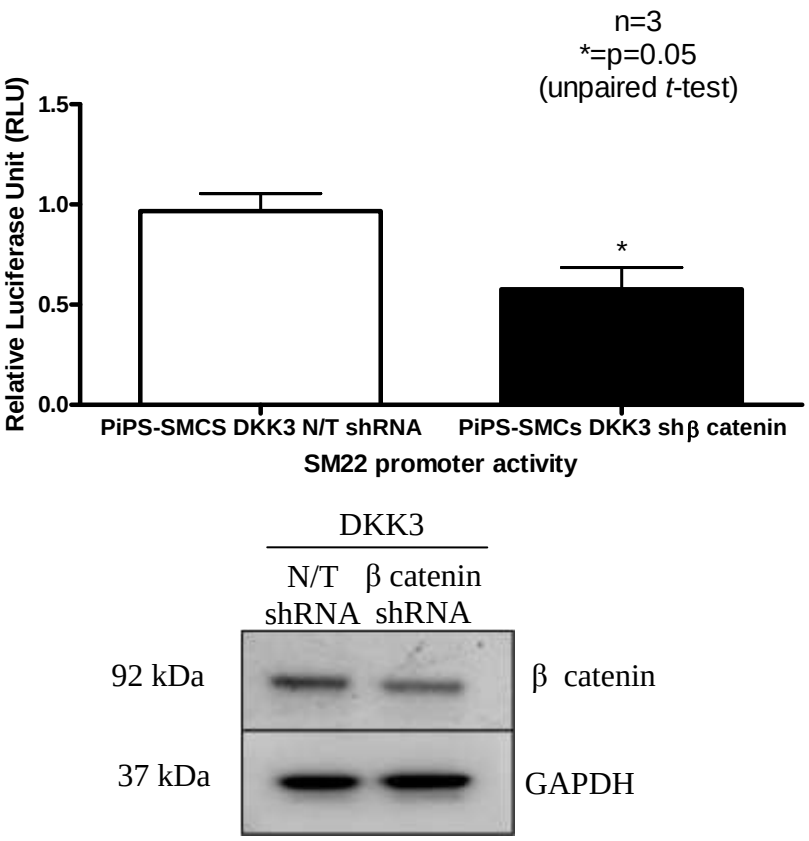


Figure 46. Transcriptional activation of SM22 by DKK3 is mediated by β catenin. (A) SM22 and TopFlash reporter activity during β catenin silencing. PiPS cells were seeded on Collagen IV and 24 hours later were viraly infected with β catenin shRNA or non targeting shRNA (10^7TU/ml complemented with 10μg/ml polybrene). 24 hours post infection the cells were forced to overexpress DKK3 (0.16μg/well), PGL3-Luc-SM22 (0.33μg/well) and PGL3-Renilla (0.1μg/well) for 48 hours after which the samples were harvested. The luciferase enzymatic activity of both samples was measured using the Lumat LB 9507 Luminometer. The enzymatic activity of Renilla comprised the internal control. Analysis of the results revealed that β catenin silencing abolished the effect of DKK3 in the promoter activity of SM22 (the mean ±SEM of 3 individual experiments is shown). **(B) Confirmation of downregulation of β catenin after lentiviral delivery of β catenin shRNA.** Western blot analysis revealed that there is a downregulation of β catenin in PiPS-SMCs-DKK3 after lentiviral delivery of β catenin shRNA. Non targeting shRNA lentiviral particles were used as a control.

3.9 DKK3 activates Wnt signalling through interaction with the transmembrane protein Kremen 1

Thus far our data have demonstrated that transcriptional activation of SM22 by DKK3 was mediated by β catenin. In order to elucidate the underlying mechanisms of this regulation, the interaction of DKK3 with proteins of the Wnt signalling pathway were assessed. It has recently been reported that DKK3 can potentiate Wnt signalling through interaction with the transmembrane protein Kremen 1 (Nakamura and Hackam, 2010). Thus, we wondered whether the potentiation of Wnt by DKK3 in our system of differentiation is mediated through interaction of DKK3 with Kremen 1.

In order to test that hypothesis, a series of co-immunoprecipitation (CoIPs) experiments were performed. Initially, an easy transfectable cell line such as HEK293-T cells was utilised to test the interaction of DKK3 and Kremen 1. DKK3 or an empty pCMV5 vector was transiently transfected in HEK293-T cells and both samples were harvested 48 hours later. Whole cell lysate was then extracted, followed by immunoprecipitation of DKK3 with a DKK3 antibody. Samples were then subjected to western blot analysis in order for binding partners of DKK3 to be identified. Immunoblotting with Kremen 1 revealed that indeed DKK3 can interact with the aforementioned receptor and interestingly an increased binding of DKK3 and Kremen 1 in the samples overexpressing DKK3 was observed (Figure 47A). These results confirm previously published data regarding the interaction of DKK3 and Kremen 1 (Nakamura and Hackam, 2010).

After establishing the interaction of DKK3 with Kremen 1 in 293-T cells, the same interaction was assessed in our system of differentiation following DKK3 stimulation. Whole cell lysate from ctl cells, PiPS-SMCs and PiPS-SMCs stimulated with 50ng/ml human recombinant DKK3 for 24 hours were subjected to immunoprecipitation using a DKK3 antibody which was followed by western blot analysis. Immunoblotting with Kremen 1 showed that while no binding between DKK3 and Kremen 1 was revealed in the ctl cells, the aforementioned proteins show binding in PiPS-SMCs and PiPS-SMCs DKK3 stimulated cells (Figure 47B). Therefore, these findings postulate that the response of PiPS-SMCs to DKK3 stimulation and the subsequent activation of Wnt signalling occurs though binding of DKK3 on Kremen 1.

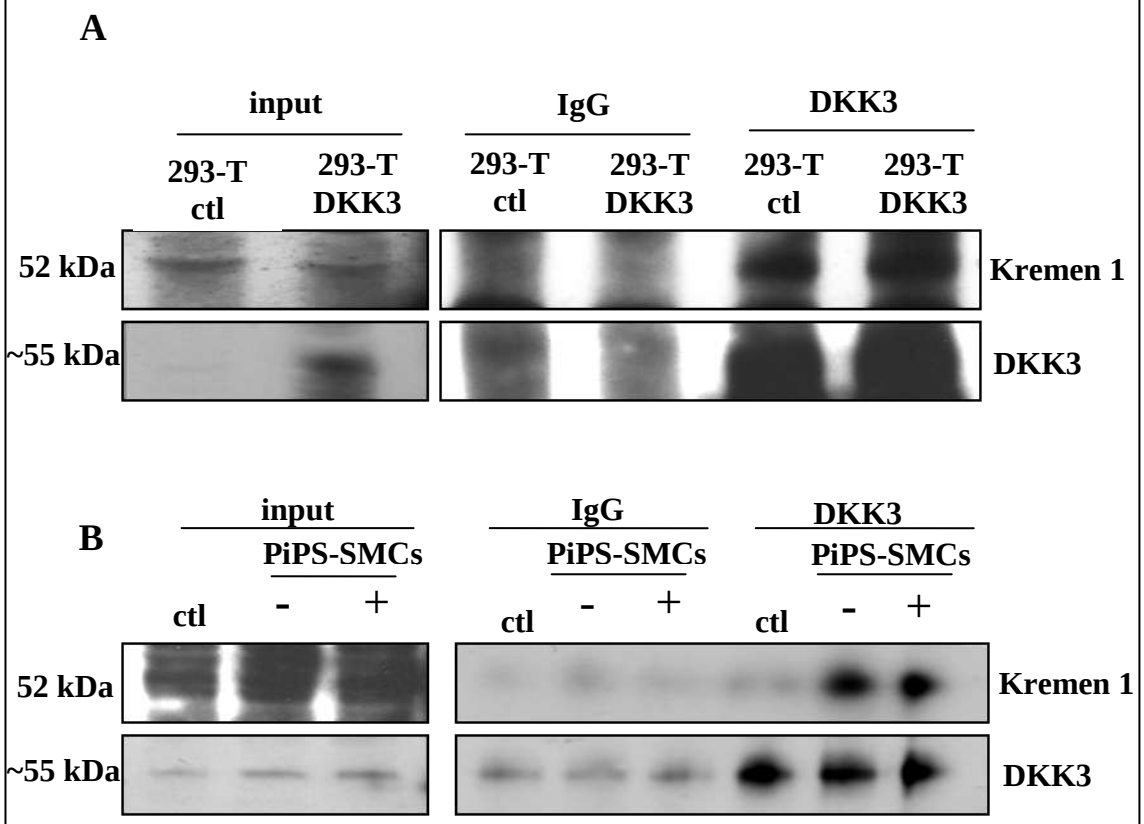


Figure 47. DKK3 binds Kremen 1 during PiPS-SMC differentiation. (A) Confirmation of DKK3 and Kremen 1 binding in HEK293-T cells. DKK3 or an empty pCMV5 vector (4µg/60% confluent T75 flask) were transiently overexpressed in HEK293-T cells for 48 hours, after which the samples were harvested and immunoprecipitated with a rabbit DKK3 antibody. Immunoblotting with Kremen 1 revealed binding of DKK3 with Kremen 1 with increased binding in the cells overexpressing DKK3. **(B) DKK3 binds on Kremen 1 during PiPS-SMCs differentiation.** Ctl and PiPS cells were seeded on Collagen IV for differentiation. 3 days later a portion of the differentiated PiPS cells were stimulated for 6 hours with 50ng/ml human recombinant DKK3. The samples were then harvested and the whole cell lysates were immunoprecipitated with a rabbit DKK3 antibody. Immunoblotting with Kremen 1 revealed that although no binding between DKK3 and Kremen 1 is revealed in the control cells, both untreated and treated PiPS-SMCs show strong binding between the aforementioned proteins.

3.10 The glycosylation status of DKK3 plays a role in PiPS-SMCs differentiation.

DKK3 is a glycosylated secreted protein with 4 glycosylation sites. Therefore we wondered whether the glycosylation status of DKK3 was important for the differentiation of PiPS cells towards SMCs. In order to examine that hypothesis, a luciferase assay as well as PNGase F deglycosylation enzyme where utilised. PiPS cells were seeded for differentiation of Collagen IV and 24 hours later were transiently transfected with a PGL3-Luc-SM22 vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well) or a TopFlash vector (0.33μg/well) and a PGL3-Renilla vector (0.1μg/well). The cells where maintained in DM for an additional 48 hour after which they were treated with a concentration of 1 unit/ml of PNGase F for 5 hours. Confirmation of successfull deglycosylation of DKK3 is shown in Figure S7. The samples were then harvested and the luciferase activity of SM22 and the Wnt reporters were measured. Analysis of the samples revealed that deglycosylation of the supernantant of PiPS-SMCs during differentiation significantly dereased the reporter activity of SM22 (Figure 48). These findings suggest that glycosylation of DKK3 is important for transcriptional regulation of SM22 during PiPS-SMCs differentiation. Furthermore, the reporter activity of TopFlash was found to be downregulated during deglycosylation of DKK3 (Figure 48) postulating that the post translational modifications of DKK3 are important for the interactions of the afforementioned protein with the Wnt signalling components, consequently affecting its activation status.

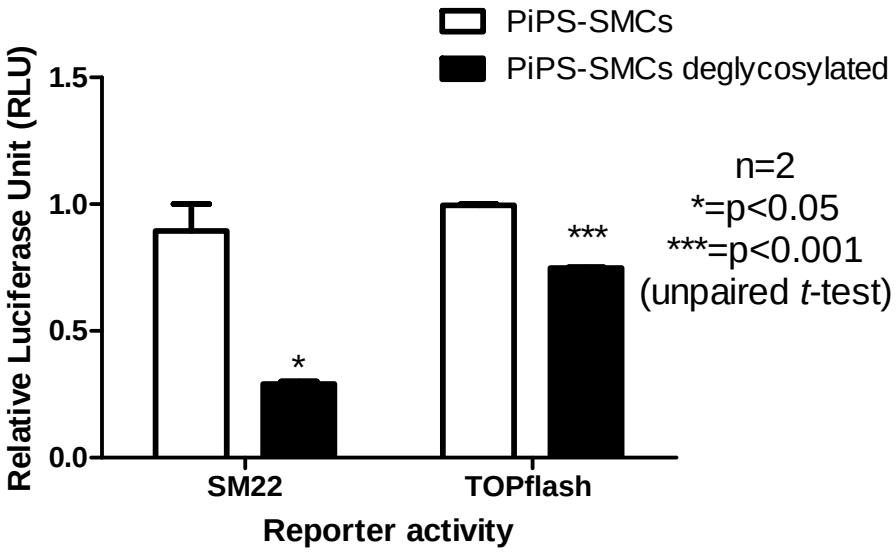


Figure 48. The reporter activity of SM22 and the Wnt target genes is affected by deglycosylation during PiPS-SMC differentiation. PiPS cells were differentiated on Collagen IV for 24 hours after which they were transciently tranfected with the reporter expression vectors PGL3-Luc-SM22 or ToPFlash (0.33μg/well) as well as a PGL3-Renilla expression vector (0.1μg/well). 48 hours later the cells were treated with 1unit/ml PNGase F deglycosylation enzyme for 5 hours after which the samples were harvested and the luciferase enzymatic activity was read. The Renilla reading comprised the interal control. The results revealed that there is a downregulation in the activity of both the SM22 reporter and the reporters of the Wnt target genes (the mean ±SEM of 2 individual experiments is shown).

3.11 PiPS-SMCs displayed SMC properties *ex vivo*

To test the functionality of the PiPS-SMCs, an *ex vivo* model generated in our lab was utilised. Fibroblasts or PiPS cells selected with neomycin (25μg/ml) were seeded on a decellularised vessel scaffold in a specially constructed bioreactor in which they were differentiated using low sheer stress, in DM containing 25ng/ml PDGF-BB. Both vessels were harvested 5 days later. The *ex vivo* PiPS-SMC vessels were then fixed and stained positive for a number of SMC markers and exibited the characteristic morphology and localization of SMCs in the media of the vessels. However, such staining was not obtained after staining of the fibroblast derived vessels (Figure 49).

Additionally, and in co-operation with Dr. Andriana Margariti, the functionality of PiPS-SMCs and PiPS derived ECs (PiPS-ECs) as well as their potential to generate functional potent vessels was investigated. A pure population of PiPS cells after selection with neomycin (25μg/ml) was seeded on a decellularized vessel in DM medium containing PDGF-BB where it was initially differentiated towards SMCs for 48 hours. The circulating medium was then exchanged to EBM-2 (endothelial specific medium) and a second seeding of pure PiPS cells was initiated. The vessels remained under constant sheer stress for 5 days after which they were harvested and fixed. Normal mouse vessels, decellularized vessles as well as the *ex vivo* double seeded vessels were then stained for Hematoxylin (HE) staining (Figure 50A). The results revealed that the re-cellularization of the vessles with PiPS-SMCs and PiPS-ECs resulted in vessels that greatly resembled a native mouse artery. Furthermore, double indirect immunofluorescence revealed a characteristing staining for the EC specific markers CD31 and CD144 and SMC markers SMA and SM22 as well as characteristic localisation of PiPS-SMCs and PiPS-ECs within the vessel scaffold (media and intima

respecitively)(Figures 50A and 50B). These results indicate the ability of PiPS cells to differentiate into both vascular lineages as well as to display vascular properties *ex vivo*. Finally, they also signify the potential of PiPS-SMCs and PiPS-ECs to generate potent functional vessles that would be capable of substituting native vessels *in vivo*.

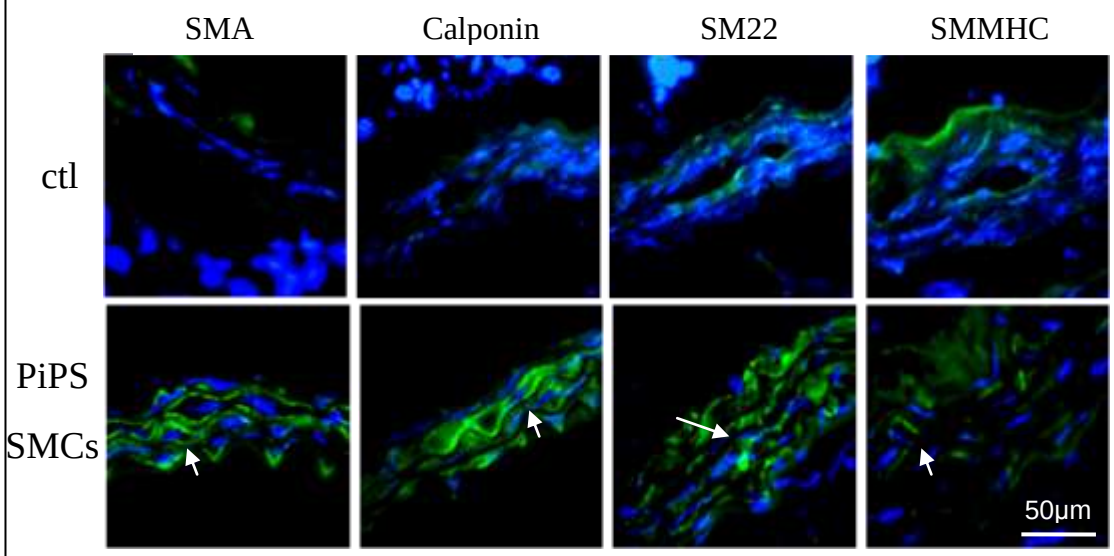


Figure 49. PiPS-SMC display SMC properties *ex vivo*. Ctl cells or PiPSs were seeded on a decellularised vessel scaffold which was connected to a specially constructed bioreactor and were differentiated for 5 days. The vessels were then harvested, fixed and stained for SMA, Calponin, SM22 and SMMHC while DAPI was used to indentify the nucelus. Indirect Immunofluorescent imaging revealed a characteristic SMC-like morphology and localization of the PiPS-SMCs within the media of the vessels. Representative cells that have incorporated withing the scafford are annotated with a white arrow. Images were captured using the Axioplan 2 fluroescent microscope at 40X magnification.

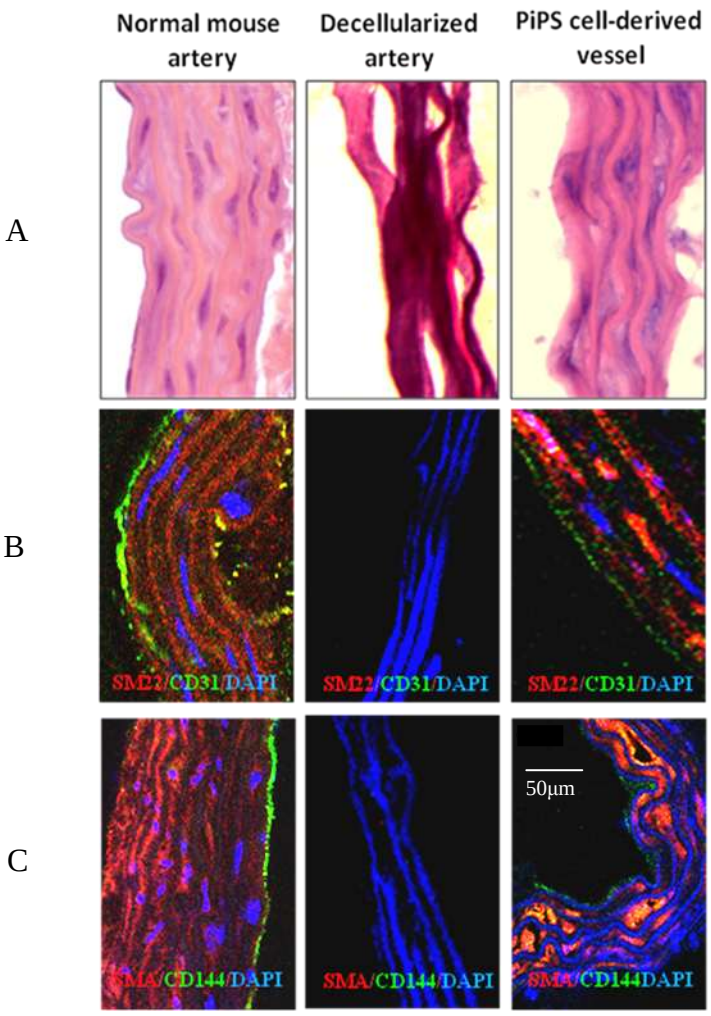


Figure 50. PiPS-SMCs and PiPS-ECs display SMC and EC properties respectively, as well as re-cellularization properties in tissue engineered vessels. (A) HE staining of the native, decellularised and double seeded tissue engineered vessels. HE staining of the double seeded vessels revealed an architecture similar to the one of the native vessel with multiple layers of SMCs and a monolayer of ECs. Successful decellularisation is confirmed by the absence of cells in the decellularised artery HE staining. **(B) and (C) Double seeded tissue engineered vessels stained positive for EC and SMC markers.** The vessels were harvested fixed and stained for SM22/CD31 and SMA/CD144. The concomitant expression of SMC and EC markers as well as characteristic morphology and localisation of PiP-SMCs and PiPS-ECs withing the media and intima respectively was revealed. Images were captured using SP5 confocal microscopy and the work was conducted in collaboration with Dr. A. Margariti.

Chapter 4
Discussion

The development of iPS cell technology has become a fascinating tool for regenerative medicine and may represent an alternative strategy to overcome the limitations that the human ESCs present with regards to therapeutic applications in humans. However, the exact molecular mechanisms that govern the process of complete transformation of a terminally differentiated somatic cell to a pluripotent one are still largely unknown.

In an attempt to elucidate the molecular changes which fibroblasts undergo on the way to pluripotency, a microarray analysis was employed in order to investigate the global gene expression of genes in different timepoints during the reprogramming. Investigation of the gene expression patterns revealed that during somatic cell reprogramming the expression of genes involved in differentiation into specific cell lineages is altered from as early as day 4. Further study of the 4 day reprogrammed cells revealed that although they display an alternate morphology and express the four reprogramming factors (SOX2, OCT4, KLF4, cMYC), they are negative for alkaline phosphatase and most importantly they do not form teratoma after transplantation *in vivo*. These cells were thus named Partially iPS or PiPS. Interestingly, although no significant changes in the expression of progenitor markers such as CD34, CD133 and cKIT were observed, the VEGFR2 (KDR) was found to be upregulated suggesting the activation of vascular associated pathways.

The potential of PiPS cells to differentiate into vascular lineages would provide the opportunity to bypass the time consuming generation of iPS cells and obtain functional cells without the tumor inducing likelihood of iPS-SMCs. Therefore, the ability of PiPS cells to differentiate toward vascular lineages was assessed by use of a standardized within our laboratory protocol of differentiation of ESCs towards SMCs (Xiao et al., 2007).

PiPS cells successfully differentiated towards SMCs expressing a subset of SMC specific markers and exhibiting a characteristic SMC-like morphology.

While trying to identify the mechanism by which PiPS cells are differentiated to SMCs, the expression of a number of genes which were differentially expressed during the reprogramming was assessed, and DKK3 was found to be upregulated in PiPS-SMCs. Investigation into the mechanism by which DKK3 could be involved in PiPS-SMC differentiation revealed that DKK3 is important for PiPS-SMC specific differentiation, Furthermore it acts as a cytokine and induces SMC specific regulation by transcriptional regulation of SM22 as revealed by Luciferase Reporter Assays.

Association of DKK3 with the Wnt signalling pathway as a member of the Dickkopf family of Wnt inhibitors lead to further investigation of the activation status of the aforementioned pathway and possible interaction of DKK3 with any of the Wnt components. We found that DKK3 can activate Wnt signalling in PiPS-SMCs leading to translocation of the pathways key mediator, β catenin, and induce Wnt specific gene expression. Furthermore, we found that DKK3 interacts with the transmembrane protein Kremen 1 in PiPS-SMCs suggesting that the activation of Wnt signalling and subsequently activation of Wnt specific expression occurs through DKK3-Kremen 1 binding.

After elucidation of the molecular mechanism by which DKK3 is regulating PiPS-SMC differentiation, *ex vivo* and *in vivo* experiments were performed in order to assess the SMC properties of the generated cells. A specially constructed bioreactor which hosted a decellularized vessel scaffold was utilised for this procedure. Seeding of the PiPS-SMCs in the decellularized scaffolds and appropriate mechanical stress from the bioreactor, lead to incorporation of the aforementioned cells into the media of the

vessels. The cells also exhibited SMC specific properties such as expression of SMC specific markers and morphology.

Finally, decellularized scaffolds which were seeded with PiPS-SMCs, and PiPS-ECs generated by Dr. Andriani Margariti were successfully generated, exhibiting properties of a potent and functional vessel *ex vivo*, similar to a native vessel. These findings demonstrate not only that PiPS cells can successfully differentiate into both vascular lineages, but they are also capable of recapitulating the properties of developmentally derived SMCs and ECs *ex vivo*.

Therefore, we have developed a method to generate PiPS cells from human fibroblasts that can differentiate into vascular cells without the risk of tumorigenesis. PiPS-SMC can be generated via a DKK3-Wnt pathway and have a great potential for tissue engineering and clinical applications in regenerative medicine.

4.1 Short term reprogramming and generation of PiPS cells

ESCs have been monopolising the field of regenerative medicine for the past decades because of their ability to be extensively expanded in culture as well as to differentiate into any cell of the 3 germ layers, making them an ideal tool for recapitulating developmental processes (Thomson et al., 1998). However, bioethical concerns, immunogenicity as well as the possibility of tumor formation rendered the cells risky for cell therapy and clinical applications (Knoepfler, 2009). Furthermore, the emergence of adult stem cells, despite their self renewal potential and their multipotential differentiation abilities which would make them ideal targets for regenerative medicine, is not without its limitations. Apart from the evident restricted capacity of differentiation when compared to ESCs, their identification and isolation can be challenging and those that can be isolated quickly become senesce *in vitro* (Digirolamo et al., 1999) or generate abnormal karyotypes (Li et al., 2007).

The generation of iPS cell technology was a breakthrough in the field of regenerative medicine. However, even though their differentiation potential seems limitless, issues such as low efficiency, tumor formation *in vivo*, random integration of retroviral vectors and the lengthy process of iPS cell line establishment, still needs to be addressed (Okita et al., 2007; Yamanaka, 2009).

Induced pluripotency is a gradual process established in a step-wise and stochastic manner. Studies with inducible vectors have shown that during reprogramming, intermediate populations that have the ability to ultimately give rise to iPS cells are generated (Hanna et al., 2009; Stadtfeld et al., 2008a). We therefore hypothesised that short term reprogramming could give rise to a cell population which would be amenable

to differentiate towards the desired cell type (in this case vascular) under the appropriate stimulus or environmental cue.

Interestingly, microarray analysis revealed that during the early stages of reprogramming a number of genes associated with differentiation of specific lineages were changed. For instance, genes associated with differentiation of connective tissue, muscle cell lines, adipocytes, bone cell, mononuclear cells, osteoclast-like cells or even adhesion of endothelial cells, were altered from as early as day 4. Furthermore, induction on day 7 of genes such as DNA (cytosine-5-)-methyltransferase 3-like (DNMT3L), a gene encoding a nuclear protein with similarities to DNA methylatransferase, reinforced the evidence by which reprogramming is supported by genome wide remodelling such as DNA methylation and histone modifications. That in turn leads to suppression or activation of specific gene subsets (Mikkelsen et al., 2008). Finally, during the later stages of reprogramming, like days 14 and 21, a series of genes associated with cell lineage differentiation were found to be altered reinforcing the hypothesis that during reprogramming, the cells exist in a transient state.

In accordance to our findings, recent studies have demonstrated that direct conversion of fibroblasts into a number of terminally differentiated cells such as blood progenitors (Szabo et al., 2010), cardiomyocytes (Efe et al., 2011; Ieda et al., 2010) and neurons (Ambasudhan et al., 2011) using defined transcription factors or direct reprogramming, is achievable and can prove to be useful for clinical applications. Specifically, Efe and his colleagues virally transduced mouse embryonic fibroblasts (MEFs) harbouring a Nebulette-LacZ reporter expressed in nascent myocardium with the four reprogramming factors and colorimetrically monitored early cardiogenesis. Their results showed that 4 days of transgenic expression of these factors were sufficient to directly reprogram

MEFs into spontaneously contracting patches of differentiated cardiomyocytes over a period of 11–12 days (Efe et al., 2011). The aforementioned studies therefore broaden the already existing field of iPS cell-factor reprogramming introducing direct reprogramming or otherwise known as lineage reprogramming as a new and promising field of regenerative medicine.

In the present study, in an attempt to investigate the transient "unstable" phase the cells enter during early reprogramming, a thorough characterisation of 4 day reprogrammed human neonatal fibroblasts (PiPS) was conducted. The four transcription factors need only be expressed transiently, thus eliminating one of the most prominent limitations of iPS cell technology such as viral integration and reactivation of transgenes thus leading to tumorigenesis (Okita et al., 2007). Most importantly, both *in vitro* and *in vivo* tests revealed that PiPS cells, as opposed to their pluripotent derivatives which form teratomas (Okita et al., 2008; Yu et al., 2007), have not yet entered the pluripotency stage and thus are unable to result to tumorigenesis even after 2 months of being transplanted into SCID mice. Finally, despite the lack of alteration in the expression patterns of vascular progenitor markers such as CD34 and cKIT, increased expression of KDR, the first molecule to be secreted during development with specificity to the endothelium (Carmeliet et al., 1996) indicates that a vascular program in PiPS cells has already been initiated after 4 days of reprogramming.

It is therefore safe to presume that during the four days of reprogramming and due to the expression of the four transcription factors, epigenetic modifications which allow the PiPS cells to enter a transient state with increased plasticity, take place. As previously shown, such epigenetic events could be demethylation of pluripotency specific genes and methylation of promoters governing somatic specific genes (Maherali et al., 2007).

It has previously been demonstrated that when stable iPS clones and aberrantly reprogrammed iPS cells were compared to ESCs, the stable iPS cell clones existed in an epigenetically similar state as ESCs whereas the semi-reprogrammed iPS cells presented with hypermethylation of pluripotency loci and were closer to MEFs (Mattout et al., 2011). Therefore, it could be suggested that in the case of PiPS, epigenetic modifications which allow the activation of cell fate determining genes may occur without being followed by activation of pluripotency loci, which is a later event during reprogramming (Stadtfeld and Hochedlinger, 2010) making the cells more susceptible to receiving specific cell lineage stimulus and differentiate into the desired cell line. Naturally, a series of experiments assessing the epigenetic status of PiPS cells would be required to elucidate the reason behind their plasticity.

The results discussed above provide for the first time a thorough characterisation of semi-reprogrammed cells, presenting a cell type that has the plasticity to directly differentiate towards a variety of cells but lacks the limitations of re-differentiated iPS cells, making it an ideal candidate for cell replacement therapy and other applications in regenerative medicine.

4.2 Differentiation of PiPS cells to SMCs

4.2.1 Collagen IV and PDGF

As previously mentioned, the microenvironment in which stem cells reside during development, determines their differentiation into different cell types. Such microenvironment cues include cytokines or growth factors, the ECM, mechanical forces and communication with adjacent cells (Xiao et al., 2010). In the present study, a model of differentiation established in our laboratory (Xiao et al., 2007) including Collagen IV and PDGF was utilised in order to induce differentiation of PiPS to SMCs.

Accumulating evidence concerning Collagen IV suggest that the aforementioned component of the ECM plays an important role in SMC differentiation from pluripotent cells. More specifically, it has been demonstrated that Sca-1^{+} progenitor cells isolated with magnetic beads form pre-differentiated ESCs could differentiate into SMCs through interaction with Collagen IV and integrin. Pre-treatment of ECS with antibodies against Collagen IV significantly inhibited SMC marker expression (Xiao et al., 2007).

Further studies have also demonstrated that Collagen IV induced the expression of mesodermal genes specific to hematopoietic, endothelial, and SMCs and also a panel of trophoectoderm-restricted markers (Schenke-Layland et al., 2007). In our study, Collagen IV seemed to favour the PiPS cell differentiation to SMCs with PiPS-SMCs expressing a number of specific SMC markers such as SMA, Calponin and SM22 reinforcing the notion that Collagen IV is a key regulator of SMC differentiation.

PDGF-BB has previously been reported to be a key regulator of SMC differentiation (Nishishita and Lin, 2004). Additional studies have also demonstrated that a high purity of SMC was obtained by ESCs (>95% of cells were positive of SMC markers) after

PDGF-BB treatment (Xiao et al., 2007). In contrast, reports showed that treatment of mature SMCs with PDGF-BB dramatically reduced SMA synthesis in the prolonged absence of serum thus suppressing SMC differentiation (Holycross et al., 1992). In the present study, no significant changes in expression of SMC specific markers were identified when PDGF-BB was omitted from the culture medium possibly suggesting that the PDGF pathway is not one of the key effectors during PiPS-SMC differentiation.

4.2.2 Generation of PiPS-SMCs

As shown above PiPS cells were successfully differentiated to SMCs using a Collagen IV/PDGF differentiation protocol. The cells expressed a panel of SMC specific markers and also exhibited a SMC-like morphology. The efficiency of PiPS-SMCs was approximately 30% reflecting the efficiency of the successfully reprogrammed cells. Upon selection of PiPS cells, the PiPS-SMCs efficiency appears to be higher (Margariti et al., 2012). Interestingly, induction of SMC marker mRNA at day 4 of PiPS cell differentiation was found to be greater than that of 6 day differentiated iPS cells under the same differentiation conditions. However, a more thorough investigation of the relative efficiency of SMC generation via the PiPS versus iPS cell is essential and is yet to be determined. Vascular SMCs play a critical role in both the physiological maintenance of the cardiovascular system as well as in the pathogenesis of vascular diseases, such as atherosclerosis, in adults (Campbell and Campbell, 1994; DeRuiter et al., 1997; Gittenberger-de Groot et al., 1999).

It has previously been shown that ESCs (Xiao et al., 2007; Xie et al., 2009), adult stem cells (Suzuki et al., 2010) and iPS cells (Moretti et al., 2010; Taura et al., 2009b) can successfully differentiate to SMCs. Although iPS cell and adult stem cell differentiation

to SMCs would provide an autologous source of SMC for patient treatment without risk of rejection, aberrant differentiation of iPS cells and danger of tumor formation as well as the need for optimization and isolation of adult stem cells and *in vitro* culturing techniques, renders them unsafe and inefficient for clinical applications.

Furthermore, the immunogenicity issue of ESC derived SMCs as well as the possibility of tumorigenesis after *in vivo* transplantation remains a concern.

As mentioned in the results section, although PiPS cells did not display changes in expression of progenitor markers such as cKIT, CD34 and CD133, the VEGFR2 (KDR) was found to be significantly upregulated after 4 days of reprogramming, suggesting a potential of these cells to differentiate into vascular cells. However, parallel studies within our laboratory have shown that PiPS can differentiate into cell lineages such as chondrocytes, adipocytes, chondrocytes and neurons in response to specific stimulus, indicating a more broad differentiation potential, beyond the limits of vascular lineages. Further studies will demonstrate the functional abilities of these cells.

Therefore, the generation of PiPS-SMCs has several implications. Initially, the time from reprogramming to obtaining SMC is significantly shorter (less than 2 weeks) when compared to the time consuming generation of iPS cells and re-differentiation to the desired cell line. This offers the opportunity to generate patient specific cells quickly and efficiently for personalised cell therapy. Additionally, one of the main concerns of stem cell and iPS cell therapy, which is tumorigenesis, is eliminated due to the fact that the purity of the differentiated cells is no longer an issue since PiPS cells do not form teratomas after subcutaneous implantation to SCID mice. Moreover, they are generated by using a non integrative method with only transient expression of the four reprogramming factors, and if required, they can be selected to obtain populations of

high purity, thus making them a more accessible and reliable source of cells for use in regenerative medicine.

As demonstrated by our *ex vivo* experiments, PiPS-SMCs hold great promise for use in tissue engineering where stem cells and iPS cells prove to be unsafe and inefficient for clinical applications. Furthermore, their potential towards vascular injury repair and cell replacement therapies is vast.

Their utilisation in mimicking SMC related genetic disorders *in vitro* as well their potential for cell based gene therapy and autologous transplantation, can revolutionise the field of disease modelling as well as tissue engineering.

It can therefore be concluded that the strategy we used here for the first time to generate PiPS-SMCs is a fast, simple, efficient and reproducible method for obtaining SMCs, which can comprise a valuable tool for regenerative medicine, cell replacement therapy as well as tissue engineering.

4.3 The role of DKK3 in PiPS-SMC differentiation

Generation of PiPS-SMCs, lead to investigation of the underlying molecular mechanism of SMC differentiation. While searching for a possible candidate responsible to induce this differentiation, the involvement of a number of genes found to be differentially expressed in the microarray analysis, was investigated during differentiation of PiPS cells to SMCs. DKK3, was found to be upregulated during PiPS-SMC differentiation in parallel with the upregulation of SMC markers. Upregulation of DKK3 lead to increase of SMC specific markers, while downregulation of DKK3 by shRNA caused a significant downregulation of SMC markers in differentiating PiPS-SMCs. These data indicate a crucial role of DKK3 in PiPS-SMC differentiation.

DKK3 is a member of the Dickkopf family of Wnt inhibitors. The hallmark of the DKK family is their ability to modulate Wnt signalling and since Wnt signalling is involved in numerous processes during development, in the adult and in disease (Cadigan and Liu, 2006; Cadigan and Nusse, 1997; Moon et al., 2004), it is not surprising that one main function of DKKs is to control cell fate in vertebrates by highly regionalized expression (Niehrs, 2006). Although DKK1 is involved in key developmental processes

such as antero-posterior (a-p) axial patterning (Glinka et al., 1998), limb formation (Mukhopadhyay et al., 2001), vertebral development (MacDonald et al., 2004) and concomitantly with DKK2 in osteoblast differentiation (van der Horst et al., 2005) as well as eye and skin development (Kazanskaya et al., 2000; Mukhopadhyay et al., 2006), DKK3 has mainly been implicated in cancer disease (Hoang et al., 2004; Kobayashi et al., 2002; Tsuji et al., 2000). Interestingly, it wasn't until recently that DKK3 was associated to tumor angiogenesis and was considered a putative pro-angiogenic factor and marker for neo angiogenesis (Zitt et al., 2008) as well as a differentiation factor involved in the remodelling of the tumor vasculature (Fong et al., 2009). Furthermore, it has been reported that elevated expression of DKK3 has been detected in tissues that mediate epithelial to mesenchymal transformation while *in situ* hybridization studies have revealed high levels of expression in the heart, brain and spinal cord. (Krupnik et al., 1999; Monaghan et al., 1999). Finally, DKK3 has been shown to modulate FGF and Activin/Nodal signalling to regulate mesoderm induction during early Xenopus development. (Pinho and Niehrs, 2007). Therefore, in the present study, demonstration of the importance of DKK3 in the differentiation towards SMCs, cells that can derive from both mesodermal and mesenchymal origins, comprises a novel role of the protein in vascular differentiation with possible developmental implications.

4.4 Underlying mechanism of DKK3-mediated SMC differentiation

In the present study, it is demonstrated that DKK3 can regulate the transcriptional patterns of SM22 via promoter activation. It is also demonstrated that DKK3 can act as a cytokine and that it can positively modulate canonical Wnt signalling and induce β catenin translocation and ultimately induction of Wnt signalling target genes. Finally, we also demonstrated that DKK3 interacts with the transmembrane protein Kremen 1 and that the glycosylation status of DKK3 is important for the transcriptional induction of SM22 as well as the activation of Wnt signalling.

In agreement with our findings, it has previously been remonstrated that Wnt3a enhances both genomic SM22 histone H3 acetylation and β catenin association, hallmarks of transcriptional activation. Analysis of a series of SM22 reporter constructs indicated that Wnt3a-regulated DNA transcriptional activation, occurred between nucleotides −213 to −192 relative to the transcription initiation site and DNA-protein complexes assembled on this element were disrupted with antibodies to β catenin, Smad2/3 and TCF7. Finally, mutation of a CAGAG motif within this region abrogated recognition by β-catenin and other DNA-binding proteins while RNAi "knockdown" of β catenin inhibited Wnt3a induction of SM22, suggesting that Wnt/β-catenin signalling controls SM22 gene transcription (Shafer and Towler, 2009). Therefore, transcriptional activation of SM22 by DKK3 in our system was postulated to occur through activation of Wnt signalling which lead to further investigation of the Wnt activation status in PiPS-SMCs.

After employment of luciferase reporter assays as well as investigation of the transcriptional levels of Wnt target genes, it was shown that Wnt signalling is activated by DKK3 in our system of PiPS-SMC differentiation. In agreement with our studies it has previously been shown by Nakamura et al. that Wnt signalling is potentiated due to upregulation of DKK3 transcripts during photoreceptor death in a mouse model of retinal degeneration. Although DKK3 potentiated Wnt signalling in Müller glia cells and HEK293 cells it failed to do so in COS7 cells (monkey kidney cells), indicating a cell type specific regulation of Wnt signalling (Nakamura et al., 2007).

As mentioned during the introduction, DKK3 is a highly N-glycosylated secretory protein (Krupnik et al., 1999). However, DKK3 specific receptors have not yet been identified. Our findings show that the levels of secreted DKK3 are elevated during differentiation to PiPS-SMC. Furthermore, stimulation of PiPS-SMC cells with human recombinant DKK3 further induced the expression of SMC specific markers suggesting that secreted DKK3 plays a role in the differentiation of PiPS to SMCs.

The role of secreted DKK3 in the induction of SMC differentiation suggests that the protein could have pharmacological implications for *in vitro* and *in vivo* applications. Recent studies have shown that adult stem cells/progenitor cells are resident within the adventitia of the vessel wall that have the potential to give rise to both endothelial and SMCs (Hu et al., 2004; Zengin et al., 2006). Evaluation of the potential of DKK3 to induce SMC differentiation of the resident, within the adventitia, progenitor cells in the form of a soluble protein could lead to the utilisation of a novel tool for directed tissue repair after injury within the vessel wall.

It has recently been demonstrated that DKK3 could selectively be internalised by EBs during the differentiation of iPS cell colonies and that this internalization was confirmed to be endocytosis (Kataoka et al., 2010). According to related studies, internalization of the DKK1/LRP5/6 complex leads to modulation of the Wnt/β-catenin pathway (Yamamoto et al., 2008), therefore the authors postulated that DKK3 could function in a similar way, thus strongly supporting the existence of a DKK3 specific receptor.

In an attempt to identify potential interacting partners of DKK3 with Wnt, Nakamura and his colleagues used a series of biochemical and functional assays to investigate the interaction between DKK3 and the Wnt pathway receptors Kremen1 (Krm1), Kremen 2 (Krm2) and low-density lipoprotein receptor-related protein 6 (LRP6). It was shown that although DKK3 can interact with both Krm1 and Krm2 it does not bind with LRP6 suggesting a novel mechanism by which DKK3 is potentiating Wnt signalling. Furthermore they demonstrated that binding of DKK3 with Kremen 1 occurred within the cells in a perinuclear pattern (Nakamura and Hackam, 2010). Since Kremens contribute to inhibition of Wnt signalling whereas LRP5/6 participates in its activation, it was postulated by the authors that the potentiation of Wnt by DKK3 could be a result of two scenarios. Firstly, isolation of the DKK3 and Kremen protein complex in intracellular membranes, such as in the ER and Golgi, resulted in reduced surface expression of Kremen receptors, a notion that was supported by previous evidence by Mao *et al* where recombinant DKK3 failed to bind to surface-expressed Kremen receptors in HEK293 cells (Mao et al., 2002). The second possibility is that DKK3 and Kremen complexes are co-expressed on the membrane surface and DKK3 physically blocks the ability of the aforementioned proteins to inhibit the Wnt pathway hence creating a favourable environment for its activation (Nakamura and Hackam, 2010).

Therefore in accordance to previously published studies, our data indicate that DKK3 potentiation of Wnt signalling during PiPS-SMC differentiation occurs through interaction of DKK3 with Kremen 1. However, further elucidation as to the localization of said interaction needs to be conducted.

Finally, in the present study, deglycosylation experiments revealed that the glycosylation status of DKK3 is important for PiPS-SMC differentiation and Wnt activation. Although it has previously been shown that the glycosylation state of DKK3 or Kremen proteins does not affect their interaction (Nakamura and Hackam, 2010), we have demonstrated by using luciferase reporter assays that treatment of PiPS-SMCs with PNGase F lead to downregulation of the SM22 promoter activity as well as the TopFlash reporter activity. These data come to add to the already diverse and controversial role of DKK3 suggesting that its deglycosylation status is context specific.

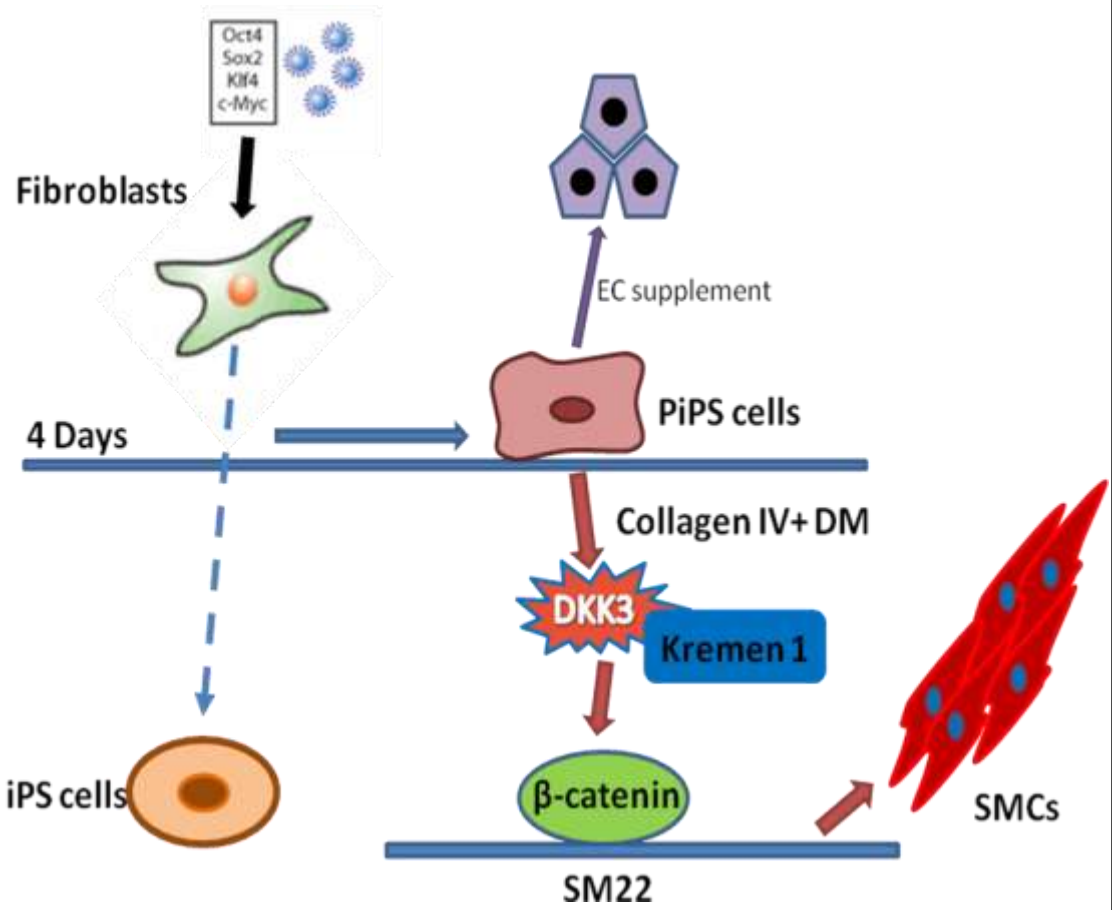


Figure 51. Schematic representation of the generation of PiPS cells as well as the mechanism by which DKK3 governs PiPS differentiation towards SMCs. PiPS cells, given the appropriate stimulus can also differentiate into functional ECs as shown in Margariti *et al*, PNAS in press.

4.5 PiPS-SMC tissue engineered vessels

One of the most promising and at the same time challenging fields of regenerative medicine is the generation of cell based tissue engineered vascular grafts that would retain the mechanical properties of native vessels *in vivo* for substantial periods of time. The vascular wall, with its unique mechanical properties, presents a challenge for tissue engineering approaches, since the source of the cells that could live up to the task is often an obstacle.

Currently, the 2 strategies used to generate tissue engineered vascular grafts are cell seeded scaffolds (L'Heureux et al., 2006; Niklason et al., 1999) and cell self-assembly (L'Heureux et al., 1998). Recent studies have shown that mesenchymal stem cells were directed to form SMC-like and EC-like cells on a decellularised ovine carodid artery which was then implanted into an ovine host and contributed to vascular tissue regeneration (Zhao et al., 2010). Furthermore, adipose derived stem cells that were shown to differentiate into SMCs were seeded in a poluglycolic acid mesh and were exposed in pulsatile stimulation in a bioreactor where it exhibited improved biomechanical properties and resembled native vessels (Wang et al., 2010). In accordance to the previously publish data, we have shown here that decellularised vessel scaffolds within a specially constructed bioreactor were seeded with PiPS cells which were forced to differentiate *ex vivo* into SMCs by application of low sheer stress. The tissue engineered vessels which were harvested and stained 5 days later revealed that the PiPS-SMCs had incorporated within the scaffold and stained positive for SMC specific markers as indicated by indirect immunofluorescent staining. Furthermore, the cells exhibited characteristic SMC-like morphology and localization within the vessel.

Similarly, double seeded decellularised vessels where the first population of cells seeded were directed to differentiate towards SMCs in DM medium within the bioreactor, whereas the second was directed to differentiate towards ECs in EMB-2 medium, were generated. In this case, a potent vessel exhibiting architecture similar to the one of the native vessel was generated. As indicated by both HE staining as well as double indirect immunofluorescent staining PiPS-SMCs occupied the media of the vessel while PiPS-ECs the incorporated within the intimal region. Recent unpublished data within our laboratory reveal that double seeded recellularised vessels engrafted into mice are patent *in vivo*, with a 60% survival rate after 3 weeks of implantation, suggesting that PiPS-SMCs and PiPS-ECs are capable of maintaining vascular stability and tone These results suggest that PiPS cells are not only capable of differentiating towards vascular lineages with SMC and EC properties *ex vivo*, but also provide a new source of cells for efficiently and rapidly generating autologous patent tissue engineered vascular grafts for application in regenerative medicine. However, comparison of *in vivo* engrafted TEVGs derived from iPS-SMCs and iPS-ECs would give us a more clear understanding of the functional differences between PiPS-SMCs and iPS-SMCs.

4.6 *In vivo* applications of PiPS-SMCs

Within the human body SMCs can be found in various tissues such as airways, the vasculature, the digestive tract and the genitourinary system. The SMCs in these tissues have the ability to contract and relax rapidly, contributing to physiological functions such as peristalsis, and simultaneously playing a critical role in the dimension change of hollow organs for regulating airflow, blood flow, organ perfusion and so on (Petschnik et al., 2010; Tanaka et al., 2008).

Therefore, all of the organs mentioned above could be potential targets for SMC replacement therapies in case of organ failure. Furthermore, SMCs could be used for cell replacement therapy in disorders such as auto-immune hepatitis (Dawkins and Joske, 1973), where anti smooth muscle antibodies are directed against actin, troponin and tropomyosin (Czaja et al., 1996) and other auto-immune disorders where SMCs could be targeted, such as Systemic lupus erythematosus, where

the body's immune system can attack the heart and blood vessels among other organs and tissues (Rahman and Isenberg, 2008).

Although SMCs for the above applications could be obtained by dissociation of blood vessels and other organs, mature SMCs exhibit limited ability for proliferation and the necessary surgical procedure may cause complications at the donor site (Suzuki et al., 2010). Therefore, PiPS-SMCs are a promising source of cells with many advantages. As opposed to stem cell and iPS cell derived SMCs, where as we have thoroughly discussed immunogenicity, tumorigenesis as well as low efficiencies and time consuming protocols comprise serious limitations for human applications, PiPS differentiation to vascular lineages constitutes a safe and efficient alternative for human *in vivo* applications.

4.7 Limitations of PiPS generation

Although the generation of PiPS cells and PiPS cell derived vascular cells comprises an easily repeatable system for the generation of cells with a striking potential for regenerative medicine, it does not come without its limitations.

The efficiency of the protocol for the generation of PiPS cells and thus their successful response to the appropriate stimulus, highly depends on the delivery of the reprogramming transcription factors within the fibroblasts. Transfection efficiencies after nucleofection of human fibroblasts in our culture usually range between 30-33% (Margariti 2012, PNAS in press) which leads to the generation of smaller population of PiPS than the one desired. Therefore, the generation of techniques that would accommodate the need for high transfection efficiencies within our system would yield larger populations of PiPS cells available for differentiation. Furthermore, the initial number of fibroblasts required for the generation of an adequate number of PiPS cells would be smaller which would prove really useful in clinical applications since smaller samples from patient biopsies would be adequate and cells will not require prolonged culture expansions.

Another concern, directly deriving from the ability to yield high nucleofection efficiencies is the purity of the PiPS cell population. Although impure populations of PiPS cells would not cause concern of tumorigenesis as we have already demonstrated during the characterization of PiPS cells (paragraph 3.2), impurity of the population would lead to lower efficiencies in the generation of differentiated cells both *in vitro* and *in vivo*. Selection with neomycin to obtain pure populations is indeed a successful solution, however, antibiotic selection can sometimes have undesired effects in the cells such as apoptosis of the transfected cells, toxicity, and decrease of proliferation (Gu et al., 1992; Kim et al., 1998).

Subsequently, bypassing technique limitations such as reprogramming efficiencies and cell selection of successfully PiPS cells, would lead to an abundant source of cells with vasculogenic potential capable of safe application in regenerative medicine.

4.8 Future work

4.8.1 Generation of PiPS cells from alternative donor cells

In the present study, we demonstrated that "plastic" cells that have the ability to differentiate towards SMCs under the appropriate stimulus can be generated by short term reprogramming after overexpression of the four reprogramming factors. It would therefore be interesting to demonstrate that application of this protocol to other terminally differentiated cells such as ECs, combined with the correct environmental cues, could lead to direct reprogramming of one somatic cell type to another. This would suggest that shot term reprogramming is a universal process not only limited to fibroblasts broadening the application of PiPS cell generation from additional somatic cell sources. Furthermore, in case of successful generation of PiPS cells from alternative sources with the same properties as our already established PiPS, it would be interesting to investigate whether application of a SMC differentiation protocol could lead to successful generation of SMC with DKK3 mediating that differentiation.

4.8.2 Comparison of PiPS-SMCs with iPS-SMCs

To further characterize the PiPS cell model, it would be informative to compare PiPS and iPS in terms of gene expression (arrays), histone modifications, as well as the differentiation potential of the two populations towards SMCs. This information will elucidate any differences in terms of incidence, plasticity, maturation and function. Moreover, cell-based assays could be performed to assess maturity and functionality of PiPS and iPS derived SMCs. Finally, the functionality of these cells should be assessed in *in vivo* experiments to highlight their potential for future clinical applications.

4.8.3 Direct generation of SMC from fibroblasts through DKK3

As it was demonstrated in the previous chapters, DKK3 is important for the induction of PiPS-SMCs by transcriptional activation of SM22 through potentiation of the Wnt/β catenin pathway. DKK3 exhibits a temporally and spatially regulated expression during development with involvement in the mesenchyme (epithelial-mesenchymal transition) as well as induction of the mesoderm (Monaghan et al., 1999; Pinho and Niehrs, 2007). It would therefore be interesting to investigate whether DKK3 could be sufficient to initiate a SMC specific program and transform fibroblasts into functional SMCs, bypassing the PiPS generation and eliminating the use of the reprogramming factors. Direct generation of SMC from one somatic cell type to another through expression of DKK3 would identify it as a global regulator of SMC differentiation, with great pharmacological potential for localised vascular repair.

4.8.4 Regulation of DKK3

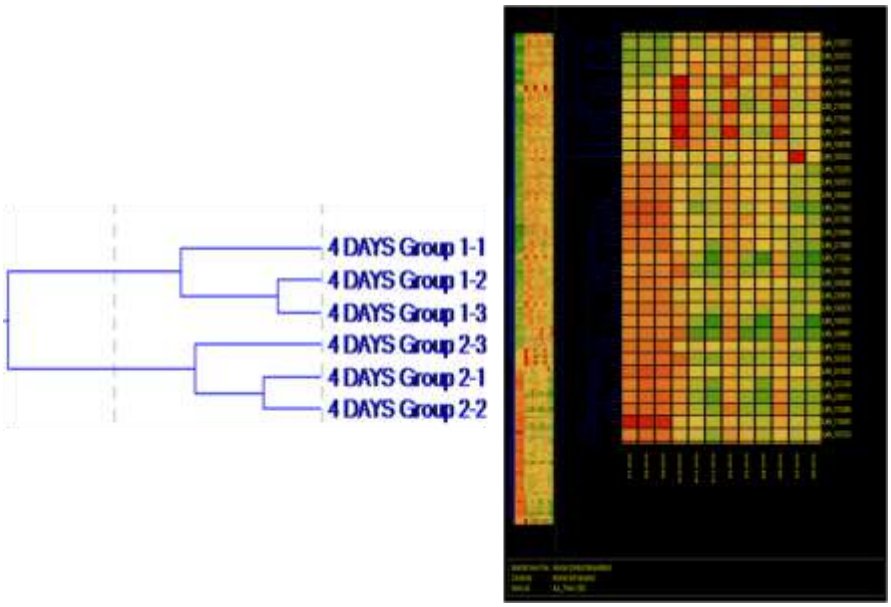
Although, DKK3 has mostly been shown to be negatively regulated by histone modifications or promoter hypermethylations in cancers (Veeck and Dahl, 2012) it has recently been demonstrated that a cluster of miRNAs regulated by MYCN inhibited the secretion of DKK3 in neuroblastoma tumors. It was also found that DKK3 was expressed in the primary neuroblastoma endothelium but was absent in the tumors expressing MYCN (Haug et al., 2011). Interestingly, very little is known about the molecular mechanisms that induce the expression of DKK3. Therefore, it would be very interesting to investigate how DKK3 is positively regulated in our system leading to induction of SMC differentiation in PiPS cells.

4.8.5 Localization of the interaction between Kremen 1 and DKK3

As previously discussed, although the interaction between Kremen 1 and DKK3 has been demonstrated, there are 2 possibilities as to where this interaction may occur (Nakamura et al., 2007). One possibility would require the interaction of the aforementioned proteins to occur in the cell surface, where DKK3 would occupy Kremen 1 receptors making them unable to inhibit Wnt signalling, thus creating a favourable environment for its activation. The second possibility would require DKK3 to bind Kremen 1 in the cytoplasm in a perinuclear pattern, inhibiting its externalization to the cell surface and yet again potentiating Wnt signalling (Nakamura et al., 2007). Since DKK3 in our study has been found to be localised in a perinuclear pattern in both ctl and PiPS-SMCs, but was also found to have a role as a cytokine during PiPS-SMC differentiation, it would be interesting to identify the localisation of DKK3-Kremen 1 interaction within our system and thus further elucidate it.

Chapter 5
Supplements

A



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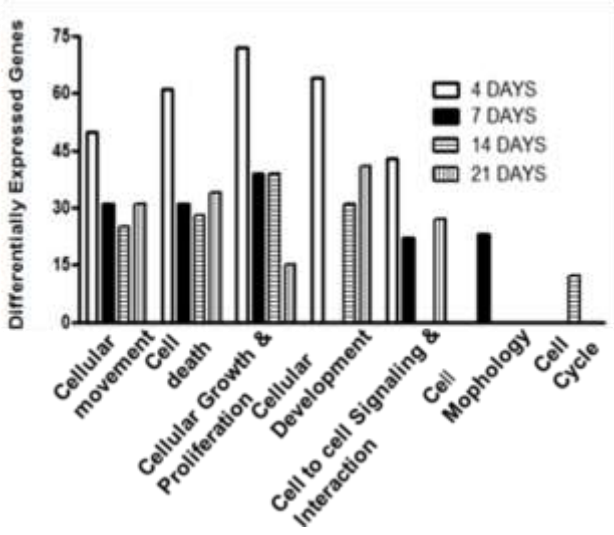


Figure S1. Microarray analysis. (A) Microarray analysis showing the differential gene expression between control cells and sample cells at 4, 7, 14, and 21 days of reprogramming. Microarray analysis was performed in triplicates. **(B) Functional classification of the genes altered at reprogramming days 4, 7, 14 and 21, analysed using the Ingenuity Systems Software.** It is noted that genes differentially expressed during cell reprogramming are involved in cell differentiation into distinct cell lineages starting as early as day 4.

Table S1: Microarray analysis

Genes differentially expressed after 4 Days of reprogramming (p<0.05)					
ProbeID	Fold	Changes	Regulation	RefSeq	ID
770615	10.464787	up	NM_002701.4	5460	POU5F1
6620170	7.6432085	up	NM_000517.3	3040	HBA2
1690066	6.730706	up	NM_002462.2	4599	MX1
5340017	6.6276045	up	NR_002196.1	283120	H19
290136	6.079792	up	NR_002304.1	5462	POU5F1P1
2570154	5.9512553	up	NM_003480.2	8076	MFAP5
110181	5.403457	down	NM_018689.1	57214	KIAA1199
270152	5.3665814	up	NM_003486.5	8140	SLC7A5
2000148	5.2977405	up	NM_001548.3	3434	IFIT1
2190674	5.295693	up	NM_000599.2	3488	IGFBP5
2120524	5.142462	up	NM_000599.2	3488	IGFBP5
4780754	5.1085076	up	XR_017655.1	645682	LOC645682
1850092	5.091201	up	NM_001018111.1	5420	PODXL
2030577	4.786988	up	NM_000362.4	7078	TIMP3
6620487	4.645717	down	NM_002993.2	6372	CXCL6
3800161	4.6291366	up	NM_005556.3	3855	KRT7
5890347	4.6051726	up	NM_017671.4	55612	FERMT1
2100767	4.537159	down	NM_130386.1	81035	COLEC12
1010246	4.3276334	up	NM_022872.2	2537	IFI6
3420605	4.305336	up	NM_007036.3	11082	ESM1
6450746	4.0860243	up	NR_002304.1	5462	POU5F1P1
6510170	4.025133	up	NM_001031683.1	3437	IFIT3
7320561	4.0224643	up	NM_016817.2	4939	OAS2
7650296	3.978758	down	NM_133505.2	1634	DCN
6480468	3.9634292	down	NM_018476.3	55859	BEX1
7550484	3.8748736	down	NM_002727.2	5552	SRGN
50368	3.8484042	down	NM_001920.3	1634	DCN
4280739	3.8371818	down	NM_153000.3	147495	APCDD1
3990170	3.8250356	up	NM_005532.3	3429	IFI27
5090215	3.7745323	down	NM_022873.2	2537	IFI6
360500	3.685463	down	NM_002727.2	5552	SRGN
3870338	3.6804771	up	NM_006820.1	10964	IFI44L
6860482	3.5597389	up	NM_017912.3	55008	HERC6
7610131	3.4570007	down	NM_001430.3	2034	EPAS1
1660673	3.3401217	down	NM_032623.2	84709	OSAP
6900408	3.3328207	down	NM_001039348.1	2202	EFEMP1
5860630	3.3157146	down	NM_016557.2	51554	CCRL1

1170709	3.3094552	up	NM_001792.2	1000	CDH2
870338	3.2915385	up	NM_001964.2	1958	EGR1
1090390	3.2486207	up	NM_001032409.1	4938	OAS1
520408	3.2206852	up	NM_001549.2	3437	IFIT3
6250563	3.2015371	down	NM_004105.3	2202	EFEMP1
6590132	3.1604493	up	NM_000598.4	3486	IGFBP3
1030333	3.1129954	down	NM_002982.3	6347	CCL2
1500280	3.1043942	up	NM_001031683.1	3437	IFIT3
6840372	3.0713813	up	NM_001013398.1	3486	IGFBP3
2940577	3.0694492	down	NM_021979.3	3306	HSPA2
2640735	3.02706	up	NM_003670.1	8553	BHLHB2
3830608	3.0222054	down	NM_032623.3	84709	OSAP
6840674	3.0089538	up	NM_007150.2	7739	ZNF185
7320669	2.9833608	down	NM_015430.2	25891	DKFZP586H2123
1070450	2.9692478	down	NM_080591.1	5742	PTGS1
1710142	2.9682157	up	NM_005613.3	5999	RGS4
7160253	2.962864	down	NM_002290.2	3910	LAMA4
1300326	2.9542146	up	NM_000459.1	7010	TEK
2600747	2.9455464	up	NM_001547.4	3433	IFIT2
1240142	2.9283273	up	NM_017654.2	54809	SAMD9
4040576	2.903735	down	NM_000600.1	3569	IL6
2100196	2.902231	up	NM_005101.1	9636	ISG15
360047	2.8936307	down	NM_002048.1	2619	GAS1
6620121	2.885797	down	NM_005623.2	6355	CCL8
7570176	2.8760169	up	NM_000459.2	7010	TEK
620102	2.8656907	up	NM_005434.3	7851	MALL
3400019	2.863984	up	NM_002923.1	5997	RGS2
870242	2.8310091	down	NM_152999.3	261729	STEAP2
4050326	2.8198087	down	NM_139125.2	5648	MASP1
1940162	2.7778716	down	NM_004120.3	2634	GBP2
580739	2.7288463	down	NM_001996.2	2192	FBLN1
290292	2.7286227	down	NM_152999.2	261729	STEAP2
6040494	2.7264404	down	NM_006487.2	2192	FBLN1
2490152	2.7000391	down	NM_001040092.1	5168	ENPP2
450189	2.6990933	up	NM_199139.1	54739	XAF1
2490364	2.6477358	down	NM_002345.3	4060	LUM
840678	2.6211014	down	NM_001040092.1	5168	ENPP2
1260040	2.6160069	down	NM_004105.3	2202	EFEMP1
5890139	2.6075144	down	NM_198148.1	119587	CPXM2
430446	2.6060336	up	NM_002281.2	3887	KRT81

2450725	2.6024663	up	NM_001013251.1	6520	SLC3A2
6580551	2.6017988	up	BX097797		
2640286	2.5986402	up	NM_000517.3	3040	HBA2
2570300	2.5933468	up	NM_006417.3	10561	IFI44
1570553	2.5871594	down	NM_000584.2	3576	IL8
1450400	2.587131	up	NM_024786.1	79844	ZDHHC11
3390372	2.568821	up	NM_001843.2	1272	CNTN1
4480035	2.5574949	up	NM_005824.1	10234	LRRC17
5090079	2.5520258	down	NM_198594.1	114897	C1QTNF1
5670674	2.5359259	down	NM_000902.3	4311	MME
1260138	2.5279663	down	XM_941135.2	651872	LOC651872
1470669	2.5154235	down	NM_002160.2	3371	TNC
7650672	2.5149841	down	NM_012098.2	23452	ANGPTL2
5420575	2.512164	up	NM_001013251.1	6520	SLC3A2
2680110	2.5071032	down	NM_014220.2	4071	TM4SF1
5700725	2.50364	up	NM_033255.2	94240	EPSTI1
4860224	2.5012639	up	NM_004184.3	7453	WARS
1340743	2.5006793	down	NM_000584.2	3576	IL8
6180554	2.4944072	down	NM_153366.2	79987	SVEP1
3460520	2.489744	up	NM_001759.2	894	CCND2
5550719	2.4882517	down	NM_133503.2	1634	DCN
6760037	2.4806926	down	NM_206929.1	54843	SYTL2
3830762	2.4640367	down	NM_181724.1	338773	TMEM119
5090671	2.4625998	up	NM_004864.1	9518	GDF15
5080139	2.4395237	up	NM_002771.2	5646	PRSS3
2480577	2.4282553	down	NM_002600.3	5142	PDE4B
4610431	2.4206407	up	NM_001615.3	72	ACTG2
5490408	2.4136531	down	NM_005195.3	1052	CEBPD
2680021	2.4058728	up	NM_018421.2	55357	TBC1D2
2060202	2.4041066	up	NM_001884.2	1404	HAPLN1
2370670	2.4029698	down	NM_001733.4	715	C1R
3420682	2.4009426	up	NM_001007139.3	3481	IGF2
2370132	2.3995693	down	NM_002546.3	4982	TNFRSF11B
7210681	2.397199	up	NM_006033.2	9388	LIPG
6860347	2.3878953	down	NM_017709.3	54855	FAM46C
1690630	2.3793766	down	NM_153690.4	131583	FAM43A
5260474	2.377786	down	NM_001733.4	715	C1R
5490470	2.3755574	up	NM_002463.1	4600	MX2
3850692	2.372314	up	NM_016270.2	10365	KLF2
5720482	2.3619733	up	NM_016323.2	51191	HERC5
4280113	2.352967	down	NM_020529.1	4792	NFKBIA
7320382	2.3403485	up	NM_030674.3	81539	SLC38A1
7150019	2.333712	down	NM_015441.1	25903	OLFML2B

6420392	2.3277297	up	NM_001014446.1	132299	OCIAD2
3930551	2.3254528	up	NM_018324.1	55301	OLAH
5420347	2.3132892	down	NM_001002018.1	54985	HCFC1R1
3780193	2.311844	up	NM_032738.3	84824	FCRLA
940274	2.311018	down	NM_000167.3	2710	GK
2970019	2.3073983	up	NM_003543.3	8365	HIST1H4H
6980458	2.2985115	down	NM_002153.1	3294	HSD17B2
770408	2.293003	up	NM_000602.1	5054	SERPINE1
4150369	2.2865605	down	NM_203391.1	2710	GK
3710068	2.2608845	up	NM_173701.1	7453	WARS
5960438	2.2539449	down	NM_139125.2	5648	MASP1
3450180	2.2480204	up	NM_002534.2	4938	OAS1
3130240	2.24696	down	NM_006206.3	5156	PDGFRA
1260669	2.2418833	down	NM_013434.4	30818	KCNIP3
5910113	2.2399476	up	NM_004385.2	1462	VCAN
4150048	2.2323222	up	NM_001444.1	2171	FABP5
4670059	2.2269552	down	NM_133646.2	51776	ZAK
3170136	2.2082808	up	NM_152703.2	219285	SAMD9L
7040035	2.1995442	up	NM_001032409.1	4938	OAS1
670386	2.197987	up	NM_181353.1	3397	ID1
5090156	2.1966922	down	NM_182744.1	4681	NBL1
3420373	2.194385	down	NM_001024466.1	6648	SOD2
6220746	2.1921287	up	NM_006851.2	11010	GLIPR1
2450435	2.1912944	down	DN997246		
1230538	2.184826	up	NM_032727.2	9118	INA
6660601	2.174433	up	NM_002133.1	3162	HMOX1
4180307	2.1695504	down	NM_014862.3	9915	ARNT2
630609	2.1676695	up	NM_022166.3	64131	XYLT1
5720356	2.1654174	up	NM_005092.2	8995	TNFSF18
6510377	2.1577802	up	NM_016639.1	51330	TNFRSF12A
3360224	2.1443355	down	NM_002421.2	4312	MMP1
1980379	2.1277287	down	NM_054110.3	117248	GALNTL2
5080543	2.1243496	up	NM_006528.2	7980	TFPI2
6660131	2.1088002	up	NM_014399.3	27075	TSPAN13
6400176	2.1042762	up	NM_004029.2	3665	IRF7
4830685	2.1023817	down	NM_006486.2	2192	FBLN1
380370	2.1020696	up	NM_016391.3	51491	HSPC111
5670465	2.1019454	down	NM_001124.1	133	ADM
3890326	2.095047	down	NM_001024465.1	6648	SOD2
10768	2.0864952	up	NM_004737.3	9215	LARGE
3310504	2.0849087	down	NM_002612.3	5166	PDK4

6840075	2.0837045	up	NM_000270.1	4860	NP
3870253	2.076963	down	NM_000609.4	6387	CXCL12
6840626	2.0762444	down	NM_000962.2	5742	PTGS1
2340072	2.0742311	up	NM_022750.2	64761	PARP12
3800537	2.0732949	up	NM_001031706.1	55041	PLEKHB2
7100010	2.0730824	down	NM_005380.4	4681	NBL1
5550220	2.067766	up	NM_006392.2	10528	NOL5A
670041	2.0630193	up	NM_144497.1	9590	AKAP12
4590370	2.0619998	up	NM_006516.1	6513	SLC2A1
7100139	2.0617127	down	NM_198392.1	6943	TCF21
10414	2.0589063	down	NM_004219.2	9232	PTTG1
6020255	2.0554085	up	NM_000399.2	1959	EGR2
5910154	2.0527813	up	NM_006392.2	10528	NOL5A
6560577	2.0508628	down	NM_006308.1	8988	HSPB3
1740291	2.0507843	down	NM_021992.2	11013	TMSL8
6650731	2.0492039	up	NM_002899.2	5947	RBP1
1510291	2.0474987	down	NM_004219.2	9232	PTTG1
7150563	2.047423	up	NM_003633.1	8507	ENC1
450491	2.043659	down	NM_033294.2	834	CASP1
5080192	2.043387	up	NM_006216.2	5270	SERPINE2
3840445	2.0425541	up	NM_000422.1	3872	KRT17
2510131	2.0401914	up	NM_004102.3	2170	FABP3
2260242	2.0369236	down	NM_057179.1	117581	TWIST2
6370541	2.034905	up	NM_001898.2	1469	CST1
6940102	2.0288663	down	NM_002160.1	3371	TNC
3180706	2.0286593	up	NM_139235.3	65083	NOL6
4390273	2.0286546	up	NM_003475.2	8045	RASSF7
2450167	2.0271757	up	NM_000992.2	6159	RPL29
240722	2.0253496	up	NM_002535.2	4939	OAS2
60121	2.0229847	down	NM_001908.3	1508	CTSB
4570441	2.0213137	up	NM_022168.2	64135	IFIH1
1980403	2.0210183	down	NM_018660.2	55893	ZNF395
2360356	2.0191083	up	NM_005860.2	10272	FSTL3
1260451	2.0167153	down	NM_198315.2	4013	VWA5A
7400707	2.0155354	down	NM_001734.2	716	C1S
4860646	2.010539	down	NM_020223.2	56975	FAM20C
990768	2.009299	up	NM_006187.2	4940	OAS3
6960379	2.0023494	down	NM_003012.3	6422	SFRP1
Genes differentially expressed after 7 Days of reprogramming (p<0.05)					
Probe	**ID**	**Fold**	**Changes**	**Regulation**	**RefSeq**
ILMN_1653934	2.192	up	XR_019109.1	650517	LOC650517
ILMN_1657039	3.192	up	NM_015687.2	27145	FILIP1
ILMN_1657234	2.456	up	NM_004591.1	6364	CCL20
ILMN_1658847	2.804	up	XM_939432.1	441478	MGC61598

ILMN_1660709	3.512	up	XR_017655.1	645682	LOC645682
ILMN_1663866	0.218	down	NM_000358.1	7045	TGFBI
ILMN_1664660	2.035	up	NM_001098409.1	645073	GAGE12G
ILMN_1666733	3.837	up	NM_000584.2	3576	IL8
ILMN_1666845	3.239	up	NM_000422.1	3872	KRT17
ILMN_1667314	4.825	up	NM_199048.1	387335	T1560
ILMN_1667796	21.05	up	NM_000517.3	3040	HBA2
ILMN_1668521	2.103	up	NM_030657.2	3982	LIM2
ILMN_1669362	3.696	up	NM_002178.2	3489	IGFBP6
ILMN_1671971	2.454	up	XR_016703.1	644743	LOC644743
ILMN_1672148	0.465	down	NM_020299.3	57016	AKR1B10
ILMN_1674009	3.23	up	NM_012253.2	8277	TKTL1
ILMN_1677038	3.272	up	NM_024913.3	79974	FLJ21986
ILMN_1677092	0.425	down	NM_181702.1	2669	GEM
ILMN_1679262	3.947	up	NM_001387.2	1809	DPYSL3
ILMN_1681260	3.054	up	XM_926633.1	643272	LOC643272
ILMN_1682636	0.464	down	NM_002089.3	2920	CXCL2
ILMN_1683067	0.424	down	XM_001134153.1	64100	ELSPBP1
ILMN_1684886	2.687	up	NM_013452.2	26609	VCX
ILMN_1688722	3.303	up	NM_000640.2	3598	IL13RA2
ILMN_1690703	2.187	up	NM_001005734.1	388815	C21orf34
ILMN_1692733	2.154	up	NM_001004354.1	441478	NRARP
ILMN_1694548	2.05	up	NM_005139.2	306	ANXA3
ILMN_1696749	2.069	up	NM_005572.3	4000	LMNA
ILMN_1699651	0.491	down	NM_000600.1	3569	IL6
ILMN_1702226	2.01	up	NM_001005732.1	388815	C21orf34
ILMN_1702585	2.628	up	XM_929774.2	646817	LOC646817
ILMN_1705546	5.11	up	NM_002701.4	5460	POU5F1
ILMN_1711087	4.634	up	XM_937579.1	648526	LOC648526
ILMN_1712774	0.386	down	NM_003604.1	8471	IRS4
ILMN_1713636	2.958	up	NM_014624.3	6277	S100A6
ILMN_1715024	2.089	up	NM_002340.3	4047	LSS
ILMN_1726448	2.083	up	NM_002421.2	4312	MMP1
ILMN_1728049	3.165	up	NM_080388.1	140576	S100A16
ILMN_1737314	0.383	down	NM_001706.2	604	BCL6
ILMN_1742025	2.092	up	NM_014279.4	10439	OLFM1
ILMN_1742332	2.522	up	NM_138444.3	115207	KCTD12
ILMN_1746085	0.186	down	NM_000598.4	3486	IGFBP3
ILMN_1748124	0.489	down	NM_198057.2	1831	TSC22D3
ILMN_1751276	0.453	down	NM_001709.3	627	BDNF
ILMN_1751576	2.215	up	NM_000459.1	7010	TEK
ILMN_1752932	2.061	up	NM_005797.2	10205	MPZL2

ILMN_1753584	2.275	up	NM_002273.2	3856	KRT8
ILMN_1753648	2.661	up	NM_032681.1	84767	SPRYD5
ILMN_1756573	2.355	up	NM_020142.3	56901	NDUFA4L2
ILMN_1759330	0.454	down	NM_004321.4	547	KIF1A
ILMN_1760199	2.7	up	NM_004929.2	793	CALB1
ILMN_1762899	0.47	down	NM_001964.2	1958	EGR1
ILMN_1764709	2.654	up	NM_005461.3	9935	MAFB
ILMN_1770505	2.168	up	NM_001197.3	638	BIK
ILMN_1772627	3.779	up	NM_001040101.1	27065	D4S234E
ILMN_1781742	2.533	up	NM_173698.1	286499	FAM133A
ILMN_1783185	2.752	up	NM_007084.2	11166	SOX21
ILMN_1784459	2.051	up	NM_002422.3	4314	MMP3
ILMN_1787186	5.496	up	NM_002514.2	4856	NOV
ILMN_1789541	2.605	up	NM_001014809.1	1400	CRMP1
ILMN_1789733	0.492	down	NM_015526.1	25999	CLIP3
ILMN_1790529	0.457	down	NM_002345.3	4060	LUM
ILMN_1791270	3.813	up	NM_006727.2	1008	CDH10
ILMN_1794742	5.159	up	NM_001010926.2	388585	HES5
ILMN_1795679	10.59	up	NM_007029.2	11075	STMN2
ILMN_1797668	2.064	up	NM_002196.2	3642	INSM1
ILMN_1801216	7.898	up	NM_005980.2	6286	S100P
ILMN_1801703	2.233	up	NM_006651.3	10815	CPLX1
ILMN_1803429	2.22	up	NM_001001391.1	960	CD44
ILMN_1803945	2.513	up	NM_006674.2	10866	HCP5
ILMN_1806165	0.424	down	NM_002155.3	3310	HSPA6
ILMN_1813131	2.115	up	XM_928128.1	643431	LOC643431
ILMN_1815673	0.472	down	NM_015881.5	27122	DKK3
ILMN_1848974	2.746	up			
ILMN_1867119	3.16	up			
ILMN_1892403	2.598	up	NR_003041.1	SNORD13	
ILMN_1907544	2.153	up			
ILMN_2059549	2.225	up	NM_003177.3	6850	SYK
ILMN_2065773	2.405	up	NM_003020.1	6447	SCG5
ILMN_2066151	2.269	up	NM_000459.2	7010	TEK
ILMN_2068498	4.059	up	NR_002304.1	POU5F1P1	
ILMN_2068499	9.667	up	NR_002304.1	POU5F1P1	
ILMN_2073758	2.193	up	NM_002426.2	4321	MMP12
ILMN_2076600	3.305	up	NM_004867.3	9452	ITM2A
ILMN_2088876	2.669	up	NM_016249.2	51438	MAGEC2
ILMN_2094266	2.434	up	NM_019089.3	54626	HES2
ILMN_2109371	3.323	up	NM_000728.3	797	CALCB
ILMN_2126038	6.47	up	NM_007029.2	11075	STMN2

ILMN_2127842	10.91	up	NM_000517.3	3040	HBA2
ILMN_2148527	3.398	up	NR_002196.1	H19	
ILMN_2150856	2.212	up	NM_002575.1	5055	SERPINB2
ILMN_2157099	2.896	up	NM_003914.2	8900	CCNA1
ILMN_2166716	2.82	up	NM_001001888.1	425054	VCX-C
ILMN_2184373	3.012	up	NM_000584.2	3576	IL8
ILMN_2188862	0.438	down	NM_004864.1	9518	GDF15
ILMN_2206126	2.364	up	NM_130900.2	154064	RAET1L
ILMN_2206746	0.392	down	NM_001711.3	633	BGN
ILMN_2219002	6.156	up	NM_005554.3	3853	KRT6A
ILMN_2276952	0.399	down	NM_004089.3	1831	TSC22D3
ILMN_2366642	2.21	up	NM_016379.2	51481	VCX3A
ILMN_2367883	0.427	down	NM_181702.1	2669	GEM
ILMN_2368530	2.464	up	NM_001012633.1	9235	IL32
ILMN_2376403	0.389	down	NM_004089.3	1831	TSC22D3
ILMN_2390310	0.382	down	NM_001001870.1	84981	C17orf91
ILMN_2396875	0.402	down	NM_001013398.1	3486	IGFBP3
ILMN_2398159	0.447	down	NM_013253.4	27122	DKK3
ILMN_2406634	2.017	up	NM_013369.2	29947	DNMT3L
ILMN_2410038	0.368	down	NM_022074.2	63901	FAM111A
Genes differentially expressed at 14 Days of reprogramming (p<0.05)					
Probe	**ID**	**Fold**	**Changes**	**Regulation**	**RefSeq**
ILMN_1655334	2.667	up	XM_939717.1	650628	LOC650628
ILMN_1656386	0.492	down	NM_014822.1	9871	SEC24D
ILMN_1657039	3.038	up	NM_015687.2	27145	FILIP1
ILMN_1658383	0.489	down	NM_002155.3	3310	HSPA6
ILMN_1658847	2.151	up	XM_939432.1	441478	MGC61598
ILMN_1660544	0.468	down	NM_183376.1	91947	ARRDC4
ILMN_1660709	4.086	up	XR_017655.1	645682	LOC645682
ILMN_1663866	0.23	down	NM_000358.1	7045	TGFBI
ILMN_1665510	0.495	down	NM_018948.2	54206	ERRFI1
ILMN_1666733	3.734	up	NM_000584.2	3576	IL8
ILMN_1666845	2.393	up	NM_000422.1	3872	KRT17
ILMN_1667314	4.081	up	NM_199048.1	387335	T1560
ILMN_1667796	17.63	up	NM_000517.3	3040	HBA2
ILMN_1669362	2.858	up	NM_002178.2	3489	IGFBP6
ILMN_1669376	0.493	down	NM_018370.2	55332	DRAM
ILMN_1670807	0.462	down	NM_174911.3	157638	FAM84B
ILMN_1672148	0.486	down	NM_020299.3	57016	AKR1B10
ILMN_1673566	0.368	down	NM_006988.3	9510	ADAMTS1
ILMN_1674009	2.277	up	NM_012253.2	8277	TKTL1
ILMN_1677038	2.633	up	NM_024913.3	79974	FLJ21986

ILMN_1677092	0.369	down	NM_181702.1	2669	GEM
ILMN_1679262	2.554	up	NM_001387.2	1809	DPYSL3
ILMN_1679984	0.407	down	NM_173798.2	170261	ZCCHC12
ILMN_1681260	2.906	up	XM_926633.1	643272	LOC643272
ILMN_1682636	0.46	down	NM_002089.3	2920	CXCL2
ILMN_1683067	0.391	down	XM_001134153.1	64100	ELSPBP1
ILMN_1683798	0.433	down	XR_001389.1	LOC404266	
ILMN_1684886	2.672	up	NM_013452.2	26609	VCX
ILMN_1687538	0.474	down	NM_005238.2	2113	ETS1
ILMN_1688722	2.498	up	NM_000640.2	3598	IL13RA2
ILMN_1690703	2.032	up	NM_001005734.1	388815	C21orf34
ILMN_1699651	0.482	down	NM_000600.1	3569	IL6
ILMN_1702585	2.121	up	XM_929774.2	646817	LOC646817
ILMN_1705546	5.795	up	NM_002701.4	5460	POU5F1
ILMN_1706028	2.003	up	NM_016378.2	51480	VCX2
ILMN_1711087	3.871	up	XM_937579.1	648526	LOC648526
ILMN_1712774	0.336	down	NM_003604.1	8471	IRS4
ILMN_1713636	2.459	up	NM_014624.3	6277	S100A6
ILMN_1728049	2.543	up	NM_080388.1	140576	S100A16
ILMN_1737314	0.309	down	NM_001706.2	604	BCL6
ILMN_1742332	2.112	up	NM_138444.3	115207	KCTD12
ILMN_1746085	0.187	down	NM_000598.4	3486	IGFBP3
ILMN_1748124	0.368	down	NM_198057.2	1831	TSC22D3
ILMN_1751276	0.459	down	NM_001709.3	627	BDNF
ILMN_1753342	0.453	down	NM_002970.1	6303	SAT1
ILMN_1753648	2.295	up	NM_032681.1	84767	SPRYD5
ILMN_1759330	0.443	down	NM_004321.4	547	KIF1A
ILMN_1760199	2.083	up	NM_004929.2	793	CALB1
ILMN_1762899	0.464	down	NM_001964.2	1958	EGR1
ILMN_1772627	2.837	up	NM_001040101.1	27065	D4S234E
ILMN_1773865	0.47	down	NM_005347.2	3309	HSPA5
ILMN_1775304	0.497	down	NM_006145.1	3337	DNAJB1
ILMN_1781742	2.111	up	NM_173698.1	286499	FAM133A
ILMN_1782305	0.499	down	NM_006186.2	4929	NR4A2
ILMN_1783185	2.108	up	NM_007084.2	11166	SOX21
ILMN_1784459	2.039	up	NM_002422.3	4314	MMP3
ILMN_1787186	3.811	up	NM_002514.2	4856	NOV
ILMN_1787265	0.482	down	NM_032772.3	84858	ZNF503
ILMN_1789733	0.48	down	NM_015526.1	25999	CLIP3
ILMN_1790529	0.423	down	NM_002345.3	4060	LUM
ILMN_1791270	3.081	up	NM_006727.2	1008	CDH10
ILMN_1791280	0.472	down	NM_014365.2	26353	HSPB8
ILMN_1793474	0.45	down	NM_198336.1	3638	INSIG1

ILMN_1794742	3.714	up	NM_001010926.2	388585	HES5
ILMN_1795679	8.444	up	NM_007029.2	11075	STMN2
ILMN_1801216	7.14	up	NM_005980.2	6286	S100P
ILMN_1806165	0.329	down	NM_002155.3	3310	HSPA6
ILMN_1815673	0.38	down	NM_015881.5	27122	DKK3
ILMN_1848974	2.256	up			
ILMN_1867119	2.703	up			
ILMN_1892403	2.124	up	NR_003041.1	SNORD13	
ILMN_2068498	4.201	up	NR_002304.1	POU5F1P1	
ILMN_2068499	8.947	up	NR_002304.1	POU5F1P1	
ILMN_2073758	2.169	up	NM_002426.2	4321	MMP12
ILMN_2076600	2.407	up	NM_004867.3	9452	ITM2A
ILMN_2088876	2.315	up	NM_016249.2	51438	MAGEC2
ILMN_2109371	2.936	up	NM_000728.3	797	CALCB
ILMN_2126038	5.374	up	NM_007029.2	11075	STMN2
ILMN_2127842	9.443	up	NM_000517.3	3040	HBA2
ILMN_2148527	2.11	up	NR_002196.1	H19	
ILMN_2157099	2.213	up	NM_003914.2	8900	CCNA1
ILMN_2166716	2.925	up	NM_001001888.1	425054	VCX-C
ILMN_2184064	0.429	down	NM_183376.1	91947	ARRDC4
ILMN_2184373	3.058	up	NM_000584.2	3576	IL8
ILMN_2188862	0.35	down	NM_004864.1	9518	GDF15
ILMN_2206126	2.062	up	NM_130900.2	154064	RAET1L
ILMN_2206746	0.335	down	NM_001711.3	633	BGN
ILMN_2215989	0.378	down	NM_005382.1	4741	NEFM
ILMN_2219002	5.339	up	NM_005554.3	3853	KRT6A
ILMN_2276952	0.342	down	NM_004089.3	1831	TSC22D3
ILMN_2309534	0.484	down	NM_001034836.1	201299	RDM1
ILMN_2366642	2.361	up	NM_016379.2	51481	VCX3A
ILMN_2367883	0.374	down	NM_181702.1	2669	GEM
ILMN_2376403	0.252	down	NM_004089.3	1831	TSC22D3
ILMN_2390310	0.415	down	NM_001001870.1	84981	C17orf91
ILMN_2396875	0.314	down	NM_001013398.1	3486	IGFBP3
ILMN_2398159	0.381	down	NM_013253.4	27122	DKK3
ILMN_2410038	0.37	down	NM_022074.2	63901	FAM111A
Genes differentially expressed after 21 Days of reprogramming (p<0.05)					
Probe	**ID**	**Fold**	**Change**	**Regulation**	**RefSeq**
ILMN_1651358	2.243	up	NM_005330.3	3046	HBE1
ILMN_1657039	3.44	up	NM_015687.2	27145	FILIP1
ILMN_1657234	2.236	up	NM_004591.1	6364	CCL20
ILMN_1658847	2.475	up	XM_939432.1	441478	MGC61598

ILMN_1660709	3.834	up	XR_017655.1	645682	LOC645682
ILMN_1663866	0.385	down	NM_000358.1	7045	TGFBI
ILMN_1664660	2.291	up	NM_001098409.1	645073	GAGE12G
ILMN_1666733	7.179	up	NM_000584.2	3576	IL8
ILMN_1667314	5.01	up	NM_199048.1	387335	T1560
ILMN_1667796	20.74	up	NM_000517.3	3040	HBA2
ILMN_1668521	2.151	up	NM_030657.2	3982	LIM2
ILMN_1669362	3.983	up	NM_002178.2	3489	IGFBP6
ILMN_1671971	2.088	up	XR_016703.1	644743	LOC644743
ILMN_1672148	0.464	down	NM_020299.3	57016	AKR1B10
ILMN_1673566	0.403	down	NM_006988.3	9510	ADAMTS1
ILMN_1674009	2.953	up	NM_012253.2	8277	TKTL1
ILMN_1674097	2.384	up	NM_001098411.1	645037	LOC645037
ILMN_1677038	4.086	up	NM_024913.3	79974	FLJ21986
ILMN_1677092	0.377	down	NM_181702.1	2669	GEM
ILMN_1677402	0.453	down	XM_941665.2	387763	LOC387763
ILMN_1679262	4.598	up	NM_001387.2	1809	DPYSL3
ILMN_1679984	0.404	down	NM_173798.2	170261	ZCCHC12
ILMN_1681260	3.616	up	XM_926633.1	643272	LOC643272
ILMN_1683067	0.398	down	XM_001134153.1	64100	ELSPBP1
ILMN_1683798	0.388	down	XR_001389.1	LOC404266	
ILMN_1684306	2.799	up	NM_019554.2	6275	S100A4
ILMN_1684886	2.889	up	NM_013452.2	26609	VCX
ILMN_1686362	2.4	up	NM_001886.1	1413	CRYBA4
ILMN_1687538	0.467	down	NM_005238.2	2113	ETS1
ILMN_1688335	2.111	up	NM_002364.3	4113	MAGEB2
ILMN_1688722	4.482	up	NM_000640.2	3598	IL13RA2
ILMN_1688780	2.036	up	NM_019554.2	6275	S100A4
ILMN_1690703	2.245	up	NM_001005734.1	388815	C21orf34
ILMN_1693242	2.086	up	NM_145288.1	162979	ZNF342
ILMN_1693650	2.031	up	NM_002005.2	2242	FES
ILMN_1696749	2.015	up	NM_005572.3	4000	LMNA
ILMN_1701783	2.05	up	NM_001922.2	1638	DCT
ILMN_1702226	2.486	up	NM_001005732.1	388815	C21orf34
ILMN_1702585	2.355	up	XM_929774.2	646817	LOC646817
ILMN_1705546	5.061	up	NM_002701.4	5460	POU5F1
ILMN_1706028	2.069	up	NM_016378.2	51480	VCX2
ILMN_1711087	4.676	up	XM_937579.1	648526	LOC648526
ILMN_1712774	0.312	down	NM_003604.1	8471	IRS4
ILMN_1713636	3.383	up	NM_014624.3	6277	S100A6
ILMN_1715024	2.1	up	NM_002340.3	4047	LSS
ILMN_1719759	0.496	down	NM_002160.2	3371	TNC

ILMN_1725311	2.08	up	NM_000160.2	2642	GCGR
ILMN_1726448	4.926	up	NM_002421.2	4312	MMP1
ILMN_1728049	2.852	up	NM_080388.1	140576	S100A16
ILMN_1737314	0.348	down	NM_001706.2	604	BCL6
ILMN_1738450	2.222	up	NM_001475.1	2577	GAGE5
ILMN_1742025	2.167	up	NM_014279.4	10439	OLFM1
ILMN_1742332	2.193	up	NM_138444.3	115207	KCTD12
ILMN_1746085	0.192	down	NM_000598.4	3486	IGFBP3
ILMN_1748124	0.343	down	NM_198057.2	1831	TSC22D3
ILMN_1751576	2.401	up	NM_000459.1	7010	TEK
ILMN_1753342	0.451	down	NM_002970.1	6303	SAT1
ILMN_1753584	2.216	up	NM_002273.2	3856	KRT8
ILMN_1753648	3.039	up	NM_032681.1	84767	SPRYD5
ILMN_1756657	2.121	up	NM_033342.2	81786	TRIM7
ILMN_1758164	2.063	up	NM_003155.2	6781	STC1
ILMN_1759330	0.479	down	NM_004321.4	547	KIF1A
ILMN_1760199	2.801	up	NM_004929.2	793	CALB1
ILMN_1762899	0.41	down	NM_001964.2	1958	EGR1
ILMN_1764709	2.445	up	NM_005461.3	9935	MAFB
ILMN_1772627	4.19	up	NM_001040101.1	27065	D4S234E
ILMN_1773079	2.082	up	NM_000090.3	1281	COL3A1
ILMN_1775336	0.485	down	NM_022117.1	64061	TSPYL2
ILMN_1775501	2.26	up	NM_000576.2	3553	IL1B
ILMN_1779252	2.507	up	NM_006074.3	10346	TRIM22
ILMN_1781742	3.033	up	NM_173698.1	286499	FAM133A
ILMN_1782705	2.048	up	NM_001475.1	2577	GAGE5
ILMN_1783185	2.911	up	NM_007084.2	11166	SOX21
ILMN_1783832	2.168	up	NM_001476.1	2578	GAGE6
ILMN_1784459	6.034	up	NM_002422.3	4314	MMP3
ILMN_1785272	2.249	up	NM_000089.3	1278	COL1A2
ILMN_1787186	6.336	up	NM_002514.2	4856	NOV
ILMN_1787265	0.453	down	NM_032772.3	84858	ZNF503
ILMN_1789541	2.626	up	NM_001014809.1	1400	CRMP1
ILMN_1790338	2.376	up	NM_016307.3	51450	PRRX2
ILMN_1791270	4.507	up	NM_006727.2	1008	CDH10
ILMN_1791280	0.456	down	NM_014365.2	26353	HSPB8
ILMN_1793474	0.415	down	NM_198336.1	3638	INSIG1
ILMN_1794742	6.073	up	NM_001010926.2	388585	HES5
ILMN_1795679	13.58	up	NM_007029.2	11075	STMN2
ILMN_1797668	2.568	up	NM_002196.2	3642	INSM1
ILMN_1801216	10.09	up	NM_005980.2	6286	S100P
ILMN_1801703	2.078	up	NM_006651.3	10815	CPLX1
ILMN_1802094	2.18	up	NM_080740.3	129025	ZNF280A

ILMN_1803429	2.263	up	NM_001001391.1	960	CD44
ILMN_1803945	2.377	up	NM_006674.2	10866	HCP5
ILMN_1806165	0.493	down	NM_002155.3	3310	HSPA6
ILMN_1848974	3.626	up			
ILMN_1867119	3.411	up			
ILMN_1891878	2.248	up			
ILMN_1892403	2.082	up	NR_003041.1	SNORD13	
ILMN_1907544	2.356	up			
ILMN_2058782	2.228	up	NM_005532.3	3429	IFI27
ILMN_2059549	2.28	up	NM_003177.3	6850	SYK
ILMN_2065773	2.521	up	NM_003020.1	6447	SCG5
ILMN_2066151	2.461	up	NM_000459.2	7010	TEK
ILMN_2068498	4.184	up	NR_002304.1	POU5F1P1	
ILMN_2068499	9.278	up	NR_002304.1	POU5F1P1	
ILMN_2076600	2.802	up	NM_004867.3	9452	ITM2A
ILMN_2088876	2.989	up	NM_016249.2	51438	MAGEC2
ILMN_2094266	2.313	up	NM_019089.3	54626	HES2
ILMN_2104356	5.059	up	NM_000089.3	1278	COL1A2
ILMN_2109371	2.605	up	NM_000728.3	797	CALCB
ILMN_2126038	7.141	up	NM_007029.2	11075	STMN2
ILMN_2127842	12.05	up	NM_000517.3	3040	HBA2
ILMN_2150851	2.127	up	NM_002575.1	5055	SERPINB2
ILMN_2150856	3.31	up	NM_002575.1	5055	SERPINB2
ILMN_2157099	2.744	up	NM_003914.2	8900	CCNA1
ILMN_2166716	3.193	up	NM_001001888.1	425054	VCX-C
ILMN_2184373	4.876	up	NM_000584.2	3576	IL8
ILMN_2188862	0.348	down	NM_004864.1	9518	GDF15
ILMN_2204876	2.694	up	NM_017791.1	55640	FLVCR2
ILMN_2206126	2.613	up	NM_130900.2	154064	RAET1L
ILMN_2215989	0.43	down	NM_005382.1	4741	NEFM
ILMN_2219002	7.372	up	NM_005554.3	3853	KRT6A
ILMN_2233576	2.201	up	NM_001477.1	26748	GAGE12I
ILMN_2276952	0.323	down	NM_004089.3	1831	TSC22D3
ILMN_2317446	2.272	up	NM_001031705.1	54967	CXorf48
ILMN_2347145	2.141	up	NM_133505.2	1634	DCN
ILMN_2366642	2.469	up	NM_016379.2	51481	VCX3A
ILMN_2367883	0.356	down	NM_181702.1	2669	GEM
ILMN_2368530	2.075	up	NM_001012633.1	9235	IL32
ILMN_2376403	0.291	down	NM_004089.3	1831	TSC22D3
ILMN_2390310	0.403	down	NM_001001870.1	84981	C17orf91
ILMN_2396875	0.35	down	NM_001013398.	3486	IGFBP3

			1		
ILMN_2406634	2.352	up	NM_013369.2	29947	DNMT3L
ILMN_2410038	0.44	down	NM_022074.2	63901	FAM111A

Microarray analysis gene description

Genes differentially expressed after 4 Days of reprogramming	
GeneSymbol	**Description**
POU5F1	Homo sapiens POU class 5 homeobox 1 (POU5F1), transcript variant 1, mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
MX1	Homo sapiens myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (MX1), mRNA.
H19	Homo sapiens H19, imprinted maternally expressed transcript (non-protein coding) (H19), non-coding RNA.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1), non-coding RNA.
MFAP5	Homo sapiens microfibrillar associated protein 5 (MFAP5), mRNA.
KIAA1199	Homo sapiens KIAA1199 (KIAA1199), mRNA.
SLC7A5	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system),member5 (SLC7A5), mRNA.
IFIT1	Homo sapiens interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), transcript 2, mRNA.
IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
LOC645682	PREDICTED: Homo sapiens hypothetical LOC645682 (LOC645682), mRNA.
PODXL	Homo sapiens podocalyxin-like (PODXL), transcript variant 1, mRNA.
TIMP3	Homo sapiens TIMP metallopeptidase inhibitor 3 (TIMP3), mRNA.
CXCL6	Homo sapiens chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) (CXCL6), mRNA.
KRT7	Homo sapiens keratin 7 (KRT7), mRNA.
FERMT1	Homo sapiens fermitin family homolog 1 (Drosophila) (FERMT1), mRNA.
COLEC12	Homo sapiens collectin sub-family member 12 (COLEC12), mRNA.
IFI6	Homo sapiens interferon, alpha-inducible protein 6 (IFI6), transcript variant 2, mRNA.
ESM1	Homo sapiens endothelial cell-specific molecule 1 (ESM1), mRNA.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1), non-coding RNA.
IFIT3	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
OAS2	Homo sapiens 2'-5'-oligoadenylate synthetase 2, 69/71kDa (OAS2), transcript variant 1, mRNA.
DCN	Homo sapiens decorin (DCN), transcript variant C, mRNA.
BEX1	Homo sapiens brain expressed, X-linked 1 (BEX1), mRNA.
SRGN	Homo sapiens serglycin (SRGN), mRNA.
DCN	Homo sapiens decorin (DCN), transcript variant A1, mRNA.
APCDD1	Homo sapiens adenomatosis polyposis coli down-regulated 1 (APCDD1), mRNA.

IFI27	Homo sapiens interferon, alpha-inducible protein 27 (IFI27), transcript variant 2, mRNA.
IFI6	Homo sapiens interferon, alpha-inducible protein 6 (IFI6), transcript variant 3, mRNA.
SRGN	Homo sapiens serglycin (SRGN), mRNA.
IFI44L	Homo sapiens interferon-induced protein 44-like (IFI44L), mRNA.
HERC6	Homo sapiens hect domain and RLD 6 (HERC6), mRNA.
EPAS1	Homo sapiens endothelial PAS domain protein 1 (EPAS1), mRNA.
OSAP	Homo sapiens ovary-specific acidic protein (OSAP), mRNA.
EFEMP1	Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), transcript 2, mRNA.
CCRL1	Homo sapiens chemokine (C-C motif) receptor-like 1 (CCRL1), transcript variant 2, mRNA.
CDH2	Homo sapiens cadherin 2, type 1, N-cadherin (neuronal) (CDH2), mRNA.
EGR1	Homo sapiens early growth response 1 (EGR1), mRNA.
OAS1	Homo sapiens 2',5'-oligoadenylate synthetase 1, 40/46kDa (OAS1), transcript variant 3, mRNA.
IFIT3	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
EFEMP1	Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), transcript 1, mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA.
CCL2	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2), mRNA.
IFIT3	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1, mRNA.
HSPA2	Homo sapiens heat shock 70kDa protein 2 (HSPA2), mRNA.
BHLHB2	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.
OSAP	Homo sapiens ovary-specific acidic protein (OSAP), mRNA.
ZNF185	Homo sapiens zinc finger protein 185 (LIM domain) (ZNF185), mRNA.
DKFZP586H2123	Homo sapiens regeneration associated muscle protease (DKFZP586H2123), transcript variant 1, mRNA.
PTGS1	Homo sapiens prostaglandin-endoperoxide synthase 1 (PTGS1), transcript variant 2, mRNA.
RGS4	Homo sapiens regulator of G-protein signalling 4 (RGS4), mRNA.
LAMA4 H	Homo sapiens laminin, alpha 4 (LAMA4), mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (venous malformations, (TEK), mRNA.
IFIT2	Homo sapiens interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA.
SAMD9	Homo sapiens sterile alpha motif domain containing 9 (SAMD9), mRNA.
IL6	Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA.
ISG15	Homo sapiens ISG15 ubiquitin-like modifier (ISG15), mRNA.
GAS1	Homo sapiens growth arrest-specific 1 (GAS1), mRNA.
CCL8	Homo sapiens chemokine (C-C motif) ligand 8 (CCL8), mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (TEK), mRNA.

MALL	Homo sapiens mal, T-cell differentiation protein-like (MALL), mRNA.
RGS2	Homo sapiens regulator of G-protein signalling 2, 24kDa (RGS2), mRNA.
STEAP2	Homo sapiens six transmembrane epithelial antigen of the prostate 2 (STEAP2), transcript variant 1, mRNA.
MASP1	Homo sapiens mannan-binding lectin serine peptidase 1 (MASP1), transcript variant 2, mRNA.
GBP2	Homo sapiens guanylate binding protein 2, interferon-inducible (GBP2), mRNA.
FBLN1	Homo sapiens fibulin 1 (FBLN1), transcript variant C, mRNA.
STEAP2	Homo sapiens six transmembrane epithelial antigen of the prostate 2 (STEAP2), mRNA.
FBLN1	Homo sapiens fibulin 1 (FBLN1), transcript variant A, mRNA.
ENPP2	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), transcript variant 2, mRNA.
XAF1	Homo sapiens XIAP associated factor 1 (XAF1), transcript variant 2, mRNA.
LUM	Homo sapiens lumican (LUM), mRNA.
ENPP2	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), transcript variant 2, mRNA.
EFEMP1	Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), transcript 1, mRNA.
CPXM2	Homo sapiens carboxypeptidase X (M14 family), member 2 (CPXM2), mRNA.
KRT81	Homo sapiens keratin 81 (KRT81), mRNA.
SLC3A2	Homo sapiens solute carrier family 3, member 2 (SLC3A2), transcript variant 6, mRNA.
BX097797	Soares_pregnant_uterus_NbHPU Homo sapiens cDNA clone IMAGp998N171164, mRNA sequence
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
IFI44	Homo sapiens interferon-induced protein 44 (IFI44), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
ZDHHC11	Homo sapiens zinc finger, DHHC-type containing 11 (ZDHHC11), mRNA.
CNTN1 H	Homo sapiens contactin 1 (CNTN1), transcript variant 1, mRNA.
LRRC17	Homo sapiens leucine rich repeat containing 17 (LRRC17), transcript variant 2, mRNA.
C1QTNF1	Homo sapiens C1q and tumor necrosis factor related protein 1 (C1QTNF1), mRNA.
MME	Homo sapiens membrane metallo-endopeptidase (MME), transcript variant 1, mRNA.
LOC651872	PREDICTED: Homo sapiens similar to C-C chemokine receptor type 11 (CCRL1) (CCX CKR) (LOC651872), mRNA.
TNC	Homo sapiens tenascin C (TNC), mRNA.
ANGPTL2	Homo sapiens angiopoietin-like 2 (ANGPTL2), mRNA.
SLC3A2	Homo sapiens solute carrier family 3, member 2 (SLC3A2), transcript variant 6, mRNA.
TM4SF1	Homo sapiens transmembrane 4 L six family member 1 (TM4SF1), mRNA.
EPSTI1	Homo sapiens epithelial stromal interaction 1 (breast) (EPSTI1), transcript variant 2, mRNA.
WARS	Homo sapiens tryptophanyl-tRNA synthetase (WARS), transcript variant 1, mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
SVEP1	Homo sapiens sushi, von Willebrand factor type A, EGF and pentraxin domain

	containing 1 (SVEP1), mRNA.
CCND2	Homo sapiens cyclin D2 (CCND2), mRNA.
DCN	Homo sapiens decorin (DCN), transcript variant A2, mRNA.
SYTL2	Homo sapiens synaptotagmin-like 2 (SYTL2), transcript variant e, mRNA.
TMEM119	Homo sapiens transmembrane protein 119 (TMEM119), mRNA.
GDF15	Homo sapiens growth differentiation factor 15 (GDF15), mRNA.
PRSS3	Homo sapiens protease, serine, 3 (mesotrypsin) (PRSS3), mRNA.
PDE4B	Homo sapiens phosphodiesterase 4B, cAMP-specific (PDE4B), transcript variant a, mRNA.
ACTG2	Homo sapiens actin, gamma 2, smooth muscle, enteric (ACTG2), mRNA.
CEBPD	Homo sapiens CCAAT/enhancer binding protein (C/EBP), delta (CEBPD), mRNA.
TBC1D2	Homo sapiens TBC1 domain family, member 2 (TBC1D2), mRNA.
HAPLN1	Homo sapiens hyaluronan and proteoglycan link protein 1 (HAPLN1), mRNA.
C1R	Homo sapiens complement component 1, r subcomponent (C1R), mRNA.
IGF2	Homo sapiens insulin-like growth factor 2 (somatomedin A) (IGF2), transcript variant 2, mRNA.
TNFRSF11B	Homo sapiens tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B), mRNA.
LIPG	Homo sapiens lipase, endothelial (LIPG), mRNA.
FAM46C	Homo sapiens family with sequence similarity 46, member C (FAM46C), mRNA.
FAM43A	Homo sapiens family with sequence similarity 43, member A (FAM43A), mRNA.
C1R	Homo sapiens complement component 1, r subcomponent (C1R), mRNA.
MX2	Homo sapiens myxovirus (influenza virus) resistance 2 (mouse) (MX2), mRNA.
KLF2	Homo sapiens Kruppel-like factor 2 (lung) (KLF2), mRNA.
HERC5	Homo sapiens hect domain and RLD 5 (HERC5), mRNA.
NFKBIA	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, (NFKBIA), mRNA.
SLC38A1	Homo sapiens solute carrier family 38, member 1 (SLC38A1), transcript variant 1, mRNA.
OLFML2B	Homo sapiens olfactomedin-like 2B (OLFML2B), mRNA.
OCIAD2	Homo sapiens OCIA domain containing 2 (OCIAD2), transcript variant 1, mRNA.
OLAH	Homo sapiens oleoyl-ACP hydrolase (OLAH), transcript variant 1, mRNA.
HCFC1R1	Homo sapiens host cell factor C1 regulator 1 (XPO1 dependent) (HCFC1R1), transcript variant 3, mRNA.
FCRLA	Homo sapiens Fc receptor-like A (FCRLA), mRNA.
GK	Homo sapiens glycerol kinase (GK), transcript variant 2, mRNA.
HIST1H4H	Homo sapiens histone cluster 1, H4h (HIST1H4H), mRNA.
HSD17B2	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2), mRNA.
SERPINE1	Homo sapiens serpin peptidase inhibitor, clade E, member 1 (SERPINE1), mRNA.
GK	Homo sapiens glycerol kinase (GK), transcript variant 1, mRNA.
WARS	Homo sapiens tryptophanyl-tRNA synthetase (WARS), transcript variant 2, mRNA.
MASP1	Homo sapiens mannan-binding lectin serine peptidase 1(MASP1), transcript variant 2, mRNA.
OAS1	Homo sapiens 2',5'-oligoadenylate synthetase 1, 40/46kDa (OAS1), transcript variant 2, mRNA.

PDGFRA	Homo sapiens platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), mRNA.
KCNIP3	Homo sapiens Kv channel interacting protein 3, calsenilin (KCNIP3), transcript variant 1, mRNA.
VCAN	Homo sapiens versican (VCAN), mRNA.
FABP5	Homo sapiens fatty acid binding protein 5 (psoriasis-associated) (FABP5), mRNA.
ZAK	Homo sapiens sterile alpha motif and leucine zipper containing kinase AZK (ZAK), transcript variant 2, mRNA.
SAMD9L	Homo sapiens sterile alpha motif domain containing 9-like (SAMD9L), mRNA.
OAS1	Homo sapiens 2',5'-oligoadenylate synthetase 1, 40/46kDa (OAS1), transcript variant 3, mRNA.
ID1	Homo sapiens inhibitor of DNA binding 1 dominant negative helix-loop-helix protein (ID1) transcript 2, mRNA.
NBL1	Homo sapiens neuroblastoma, suppression of tumorigenicity 1 (NBL1), transcript variant 1, mRNA.
SOD2	Homo sapiens superoxide dismutase 2, mitochondrial (SOD2), transcript variant 3, mRNA.
GLIPR1	Homo sapiens GLI pathogenesis-related 1 (GLIPR1), mRNA.
TC125227	Human breast cancer tissue, large insert, pCMV expression (LOC387724), mRNA sequence
INA	Homo sapiens internexin neuronal intermediate filament protein, alpha (INA), mRNA.
HMOX1	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA.
ARNT2	Homo sapiens aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2), mRNA.
XYLT1	Homo sapiens xylosyltransferase I (XYLT1), mRNA.
TNFSF18	Homo sapiens tumor necrosis factor (ligand) superfamily, member 18 (TNFSF18), mRNA.
TNFRSF12A	Homo sapiens tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A), mRNA.
MMP1	Homo sapiens matrix metallopeptidase 1 (interstitial collagenase) (MMP1), mRNA.
GALNTL2	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine: (GALNTL2), mRNA.
TFPI2	Homo sapiens tissue factor pathway inhibitor 2 (TFPI2), mRNA.
TSPAN13	Homo sapiens tetraspanin 13 (TSPAN13), mRNA.
IRF7	Homo sapiens interferon regulatory factor 7 (IRF7), transcript variant b, mRNA.
FBLN1	Homo sapiens fibulin 1 (FBLN1), transcript variant D, mRNA.
HSPC111	Homo sapiens hypothetical protein HSPC111 (HSPC111), mRNA.
ADM	Homo sapiens adrenomedullin (ADM), mRNA.
SOD2	Homo sapiens superoxide dismutase 2, mitochondrial (SOD2), transcript variant 2, mRNA.
LARGE	Homo sapiens like-glycosyltransferase (LARGE), transcript variant 1, mRNA.
PDK4	Homo sapiens pyruvate dehydrogenase kinase, isozyme 4 (PDK4), mRNA.
NP	Homo sapiens nucleoside phosphorylase (NP), mRNA.
CXCL12	Homo sapiens chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), transcript variant 2, mRNA.
PTGS1	Homo sapiens prostaglandin-endoperoxide synthase 1 (PTGS1), transcript variant 1, mRNA.

PARP12	Homo sapiens poly (ADP-ribose) polymerase family, member 12 (PARP12), mRNA.
PLEKHB2	Homo sapiens pleckstrin homology domain containing, family B (evectins) (PLEKHB2), transcript 1, mRNA.
NBL1	Homo sapiens neuroblastoma, suppression of tumorigenicity 1 (NBL1), transcript variant 2, mRNA.
NOL5A	Homo sapiens nucleolar protein 5A (56kDa with KKE/D repeat) (NOL5A), mRNA.
AKAP12	Homo sapiens A kinase (PRKA) anchor protein (gravin) 12 (AKAP12), transcript variant 2, mRNA.
SLC2A1	Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA.
TCF21	Homo sapiens transcription factor 21 (TCF21), transcript variant 1, mRNA.
PTTG1	Homo sapiens pituitary tumor-transforming 1 (PTTG1), mRNA.
EGR2	Homo sapiens early growth response 2 (Krox-20 homolog, Drosophila) (EGR2), mRNA.
NOL5A	Homo sapiens nucleolar protein 5A (56kDa with KKE/D repeat) (NOL5A), mRNA.
HSPB3	Homo sapiens heat shock 27kDa protein 3 (HSPB3), mRNA.
TMSL8	Homo sapiens thymosin-like 8 (TMSL8), mRNA.
RBP1	Homo sapiens retinol binding protein 1, cellular (RBP1), mRNA.
PTTG1	Homo sapiens pituitary tumor-transforming 1 (PTTG1), mRNA.
ENC1	Homo sapiens ectodermal-neural cortex (with BTB-like domain) (ENC1), mRNA.
CASP1	Homo sapiens caspase 1, apoptosis-related cysteine peptidase (CASP1), transcript variant delta, mRNA.
SERPINE2	Homo sapiens serpin peptidase inhibitor, clade E , member 2 (SERPINE2), mRNA.
KRT17	Homo sapiens keratin 17 (KRT17), mRNA.
FABP3	Homo sapiens fatty acid binding protein 3, muscle and heart (FABP3), mRNA.
TWIST2	Homo sapiens twist homolog 2 (Drosophila) (TWIST2), mRNA.
CST1	Homo sapiens cystatin SN (CST1), mRNA.
TNC	Homo sapiens tenascin C (hexabrachion) (TNC), mRNA.
NOL6	Homo sapiens nucleolar protein family 6 (RNA-associated) (NOL6), transcript variant gamma, mRNA.
RASSF7	Homo sapiens Ras association (RalGDS/AF-6) domain family (N-terminal) member 7 (RASSF7), mRNA.
RPL29	Homo sapiens ribosomal protein L29 (RPL29), mRNA.
OAS2	Homo sapiens 2'-5'-oligoadenylate synthetase 2, 69/71kDa (OAS2), transcript variant 2, mRNA.
CTSB	Homo sapiens cathepsin B (CTSB), transcript variant 1, mRNA.
IFIH1	Homo sapiens interferon induced with helicase C domain 1 (IFIH1), mRNA.
ZNF395	Homo sapiens zinc finger protein 395 (ZNF395), mRNA.
FSTL3	Homo sapiens follistatin-like 3 (secreted glycoprotein) (FSTL3), mRNA.
VWA5A	Homo sapiens von Willebrand factor A domain containing 5A (VWA5A), transcript variant 2, mRNA.
C1S	Homo sapiens complement component 1, s subcomponent (C1S), transcript variant 1, mRNA.
FAM20C	Homo sapiens family with sequence similarity 20, member C (FAM20C), mRNA.
OAS3	Homo sapiens 2'-5'-oligoadenylate synthetase 3, 100kDa (OAS3), mRNA.
SFRP1	Homo sapiens secreted frizzled-related protein 1 (SFRP1), mRNA.

Genes differentially expressed after 7 Days of reprogramming	
GeneSymbol	**Description**
LOC650517	PREDICTED: Homo sapiens hypothetical LOC650517 (LOC650517), mRNA.
FILIP1	Homo sapiens filamin A interacting protein 1 (FILIP1), mRNA.
CCL20	Homo sapiens chemokine (C-C motif) ligand 20 (CCL20), mRNA.
MGC61598.	PREDICTED: Homo sapiens similar to ankyrin-repeat protein Nrarp (MGC61598), mRNA
LOC645682	PREDICTED: Homo sapiens hypothetical LOC645682 (LOC645682), mRNA.
TGFBI	Homo sapiens transforming growth factor, beta-induced, 68kDa (TGFBI), mRNA.
GAGE12G	Homo sapiens G antigen 12G (GAGE12G), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
KRT17	Homo sapiens keratin 17 (KRT17), mRNA.
T1560	Homo sapiens T1560 protein (T1560), mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
LIM2	Homo sapiens lens intrinsic membrane protein 2, 19kDa (LIM2), mRNA.
IGFBP6	Homo sapiens insulin-like growth factor binding protein 6 (IGFBP6), mRNA.
LOC644743	PREDICTED: Homo sapiens hypothetical LOC644743 (LOC644743), mRNA.
AKR1B10	Homo sapiens aldo-keto reductase family 1, member B10 (aldose reductase) (AKR1B10), mRNA.
TKTL1	Homo sapiens transketolase-like 1 (TKTL1), mRNA.
FLJ21986	Homo sapiens hypothetical protein FLJ21986 (FLJ21986), mRNA.
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
DPYSL3	Homo sapiens dihydropyrimidinase-like 3 (DPYSL3), mRNA.
LOC643272	PREDICTED: Homo sapiens hypothetical protein LOC643272 (LOC643272), mRNA.
CXCL2	Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA.
ELSPBP1	PREDICTED: Homo sapiens epididymal sperm binding protein 1 (ELSPBP1), mRNA.
VCX	Homo sapiens variable charge, X-linked (VCX), mRNA.
IL13RA2	Homo sapiens interleukin 13 receptor, alpha 2 (IL13RA2), mRNA.
C21orf34	Homo sapiens chromosome 21 open reading frame 34 (C21orf34), transcript variant 3, mRNA.
NRARP	Homo sapiens Notch-regulated ankyrin repeat protein (NRARP), mRNA.
ANXA3	Homo sapiens annexin A3 (ANXA3), mRNA.
LMNA	Homo sapiens lamin A/C (LMNA), transcript variant 2, mRNA.
IL6	Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA.
C21orf34	Homo sapiens chromosome 21 open reading frame 34 (C21orf34), transcript variant 1, mRNA.
LOC646817	PREDICTED: Homo sapiens similar to Protein SET (LOC646817), mRNA.
POU5F1	Homo sapiens POU class 5 homeobox 1 (POU5F1), transcript variant 1, mRNA.
LOC648526	PREDICTED: Homo sapiens similar to epiplakin 1 (LOC648526), mRNA.
IRS4	Homo sapiens insulin receptor substrate 4 (IRS4), mRNA.
S100A6	Homo sapiens S100 calcium binding protein A6 (S100A6), mRNA.
LSS	Homo sapiens lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS),

	transcript variant 1, mRNA.
MMP1	Homo sapiens matrix metallopeptidase 1 (interstitial collagenase) (MMP1), mRNA.
S100A16	Homo sapiens S100 calcium binding protein A16 (S100A16), mRNA.
BCL6	Homo sapiens B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6), transcript variant 1, mRNA.
OLFM1	Homo sapiens olfactomedin 1 (OLFM1), transcript variant 1, mRNA.
KCTD12	Homo sapiens potassium channel tetramerisation domain containing 12 (KCTD12), mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 1, mRNA.
BDNF	Homo sapiens brain-derived neurotrophic factor (BDNF), transcript variant 4, mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (TEK), mRNA.
MPZL2	Homo sapiens myelin protein zero-like 2 (MPZL2), transcript variant 1, mRNA.
KRT8 .	Homo sapiens keratin 8 (KRT8), mRNA
SPRYD5	Homo sapiens SPRY domain containing 5 (SPRYD5), mRNA.
NDUFA4L2	Homo sapiens NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (NDUFA4L2), mRNA.
KIF1A	Homo sapiens kinesin family member 1A (KIF1A), mRNA.
CALB1	Homo sapiens calbindin 1, 28kDa (CALB1), mRNA.
EGR1	Homo sapiens early growth response 1 (EGR1), mRNA.
MAFB	Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) (MAFB), mRNA.
BIK	Homo sapiens BCL2-interacting killer (apoptosis-inducing) (BIK), mRNA.
D4S234E	Homo sapiens DNA segment on chromosome 4 (unique) 234 expressed seq (D4S234E), transcript2, mRNA.
FAM133A	Homo sapiens family with sequence similarity 133, member A (FAM133A), mRNA.
SOX21	Homo sapiens SRY (sex determining region Y)-box 21 (SOX21), mRNA.
MMP3	Homo sapiens matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3), mRNA.
NOV	Homo sapiens nephroblastoma overexpressed gene (NOV), mRNA.
CRMP1	Homo sapiens collapsin response mediator protein 1 (CRMP1), transcript variant 1, mRNA.
CLIP3	Homo sapiens CAP-GLY domain containing linker protein 3 (CLIP3), mRNA.
LUM	Homo sapiens lumican (LUM), mRNA.
CDH10	Homo sapiens cadherin 10, type 2 (T2-cadherin) (CDH10), mRNA.
HES5	Homo sapiens hairy and enhancer of split 5 (Drosophila) (HES5), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.
INSM1	Homo sapiens insulinoma-associated 1 (INSM1), mRNA.
S100P	Homo sapiens S100 calcium binding protein P (S100P), mRNA.
CPLX1	Homo sapiens complexin 1 (CPLX1), mRNA.
CD44	Homo sapiens CD44 molecule (Indian blood group) (CD44), transcript variant 4, mRNA.
HCP5	Homo sapiens HLA complex P5 (HCP5), mRNA.

HSPA6	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.
LOC643431	PREDICTED: Homo sapiens similar to Keratin, type II cytoskeletal 8 (Cytokeratin-8) (K8) (LOC643431), mRNA.
DKK3	Homo sapiens dickkopf homolog 3 (Xenopus laevis) (DKK3), transcript variant 1, mRNA.
	qk01b06.x1 NCI_CGAP_Kid3 Homo sapiens cDNA clone IMAGE:1867667 3, mRNA sequence
	EST10539 Adipose tissue, white I Homo sapiens cDNA 5 end, mRNA sequence
SNORD13	Homo sapiens small nucleolar RNA, C/D box 13 (SNORD13) on chromosome 8.
	UI-E-EJ1-ajw-b-16-0-UI.r1 UI-E-EJ1 Homo sapiens cDNA clone UI-E-EJ1-ajw-b-16-0-UI 5, mRNA sequence
SYK	Homo sapiens spleen tyrosine kinase (SYK), mRNA.
SCG5	Homo sapiens secretogranin V (7B2 protein) (SCG5), mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (TEK), mRNA.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
MMP12	Homo sapiens matrix metallopeptidase 12 (macrophage elastase) (MMP12), mRNA.
ITM2A	Homo sapiens integral membrane protein 2A (ITM2A), mRNA.
MAGEC2	Homo sapiens melanoma antigen family C, 2 (MAGEC2), mRNA.
HES2	Homo sapiens hairy and enhancer of split 2 (Drosophila) (HES2), mRNA.
CALCB	Homo sapiens calcitonin-related polypeptide beta (CALCB), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
H19	Homo sapiens H19, imprinted maternally expressed transcript (H19) on chromosome 11.
SERPINB2	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), mRNA.
CCNA1	Homo sapiens cyclin A1 (CCNA1), mRNA.
VCX-C	Homo sapiens variably charged X-C (VCX-C), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
GDF15	Homo sapiens growth differentiation factor 15 (GDF15), mRNA.
RAET1L	Homo sapiens retinoic acid early transcript 1L (RAET1L), mRNA.
BGN	Homo sapiens biglycan (BGN), mRNA.
KRT6A	Homo sapiens keratin 6A (KRT6A), mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.
VCX3A	Homo sapiens variable charge, X-linked 3A (VCX3A), mRNA.
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
IL32	Homo sapiens interleukin 32 (IL32), transcript variant 4, mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.
C17orf91	Homo sapiens chromosome 17 open reading frame 91 (C17orf91), transcript variant 2, mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript

	variant 1, mRNA.
DKK3	Homo sapiens dickkopf homolog 3 (Xenopus laevis) (DKK3), transcript variant 2, mRNA.
DNMT3L	Homo sapiens DNA (cytosine-5-)-methyltransferase 3-like (DNMT3L), transcript variant 1, mRNA.
FAM111A	Homo sapiens family with sequence similarity 111, member A (FAM111A), transcript variant 1, mRNA.
Genes differentially expressed after 1 4 Days of reprogramming	
GeneSymbol	**Description**
LOC650628	PREDICTED: Homo sapiens hypothetical protein LOC650628 (LOC650628), mRNA.
SEC24D	Homo sapiens SEC24 related gene family, member D (S. cerevisiae) (SEC24D), mRNA.
FILIP1	Homo sapiens filamin A interacting protein 1 (FILIP1), mRNA.
HSPA6	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.
MGC61598	PREDICTED: Homo sapiens similar to ankyrin-repeat protein Nrarp (MGC61598), mRNA.
ARRDC4	Homo sapiens arrestin domain containing 4 (ARRDC4), mRNA.
LOC645682	PREDICTED: Homo sapiens hypothetical LOC645682 (LOC645682), mRNA.
TGFBI	Homo sapiens transforming growth factor, beta-induced, 68kDa (TGFBI), mRNA.
ERRFI1	Homo sapiens ERBB receptor feedback inhibitor 1 (ERRFI1), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
KRT17	Homo sapiens keratin 17 (KRT17), mRNA.
T1560	Homo sapiens T1560 protein (T1560), mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
IGFBP6	Homo sapiens insulin-like growth factor binding protein 6 (IGFBP6), mRNA.
DRAM	Homo sapiens damage-regulated autophagy modulator (DRAM), mRNA.
FAM84B	Homo sapiens family with sequence similarity 84, member B (FAM84B), mRNA.
AKR1B10	Homo sapiens aldo-keto reductase family 1, member B10 (aldose reductase) (AKR1B10), mRNA.
ADAMTS1	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS1), mRNA.
TKTL1	Homo sapiens transketolase-like 1 (TKTL1), mRNA.
FLJ21986	Homo sapiens hypothetical protein FLJ21986 (FLJ21986), mRNA.
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
DPYSL3	Homo sapiens dihydropyrimidinase-like 3 (DPYSL3), mRNA.
ZCCHC12	Homo sapiens zinc finger, CCHC domain containing 12 (ZCCHC12), mRNA.
LOC643272	PREDICTED: Homo sapiens hypothetical protein LOC643272 (LOC643272), mRNA.
CXCL2	Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA.
ELSPBP1	PREDICTED: Homo sapiens epididymal sperm binding protein 1 (ELSPBP1), mRNA.
LOC404266	PREDICTED: Homo sapiens hypothetical LOC404266 (LOC404266), misc RNA.
VCX	Homo sapiens variable charge, X-linked (VCX), mRNA.
ETS1	Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)

	(ETS1), mRNA.
IL13RA2	Homo sapiens interleukin 13 receptor, alpha 2 (IL13RA2), mRNA.
C21orf34	Homo sapiens chromosome 21 open reading frame 34 (C21orf34), transcript variant 3, mRNA.
IL6	Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA.
LOC646817	PREDICTED: Homo sapiens similar to Protein SET (LOC646817), mRNA.
POU5F1	Homo sapiens POU class 5 homeobox 1 (POU5F1), transcript variant 1, mRNA.
VCX2	Homo sapiens variable charge, X-linked 2 (VCX2), mRNA.
LOC648526	PREDICTED: Homo sapiens similar to epiplakin 1 (LOC648526), mRNA.
IRS4	Homo sapiens insulin receptor substrate 4 (IRS4), mRNA.
S100A6	Homo sapiens S100 calcium binding protein A6 (S100A6), mRNA.
S100A16	Homo sapiens S100 calcium binding protein A16 (S100A16), mRNA.
BCL6	Homo sapiens B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6), transcript variant 1, mRNA.
KCTD12	Homo sapiens potassium channel tetramerisation domain containing 12 (KCTD12), mRNA.
IGFBP3 .	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 1, mRNA.
BDNF	Homo sapiens brain-derived neurotrophic factor (BDNF), transcript variant 4, mRNA.
SAT1	Homo sapiens spermidine/spermine N1-acetyltransferase 1 (SAT1), mRNA.
SPRYD5	Homo sapiens SPRY domain containing 5 (SPRYD5), mRNA.
KIF1A	Homo sapiens kinesin family member 1A (KIF1A), mRNA.
CALB1	Homo sapiens calbindin 1, 28kDa (CALB1), mRNA.
EGR1	Homo sapiens early growth response 1 (EGR1), mRNA.
D4S234E .	Homo sapiens DNA segment on chromosome 4 (unique) 234 expressed sequence (D4S234E), transc 2, mRNA
HSPA5	Homo sapiens heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (HSPA5), mRNA.
DNAJB1	Homo sapiens DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1), mRNA.
FAM133A	Homo sapiens family with sequence similarity 133, member A (FAM133A), mRNA.
NR4A2	Homo sapiens nuclear receptor subfamily 4, group A, member 2 (NR4A2), transcript variant 1, mRNA.
SOX21	Homo sapiens SRY (sex determining region Y)-box 21 (SOX21), mRNA.
MMP3	Homo sapiens matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3), mRNA.
NOV	Homo sapiens nephroblastoma overexpressed gene (NOV), mRNA.
ZNF503	Homo sapiens zinc finger protein 503 (ZNF503), mRNA.
CLIP3	Homo sapiens CAP-GLY domain containing linker protein 3 (CLIP3), mRNA.
LUM	Homo sapiens lumican (LUM), mRNA.
CDH10	Homo sapiens cadherin 10, type 2 (T2-cadherin) (CDH10), mRNA.
HSPB8	Homo sapiens heat shock 22kDa protein 8 (HSPB8), mRNA.
INSIG1	Homo sapiens insulin induced gene 1 (INSIG1), transcript variant 2, mRNA.
HES5	Homo sapiens hairy and enhancer of split 5 (Drosophila) (HES5), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.

S100P	Homo sapiens S100 calcium binding protein P (S100P), mRNA.
HSPA6	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.
DKK3	Homo sapiens dickkopf homolog 3 (Xenopus laevis) (DKK3), transcript variant 1, mRNA.
	qk01b06.x1 NCI_CGAP_Kid3 Homo sapiens cDNA clone IMAGE:1867667 3, mRNA sequence
	EST10539 Adipose tissue, white I Homo sapiens cDNA 5 end, mRNA sequence
	SNORD13 Homo sapiens small nucleolar RNA, C/D box 13 (SNORD13) on chromosome 8.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
MMP12	Homo sapiens matrix metallopeptidase 12 (macrophage elastase) (MMP12), mRNA.
ITM2A	Homo sapiens integral membrane protein 2A (ITM2A), mRNA.
MAGEC2	Homo sapiens melanoma antigen family C, 2 (MAGEC2), mRNA.
CALCB	Homo sapiens calcitonin-related polypeptide beta (CALCB), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
H19	Homo sapiens H19, imprinted maternally expressed transcript (H19) on chromosome 11.
CCNA1	Homo sapiens cyclin A1 (CCNA1), mRNA.
VCX-C	Homo sapiens variably charged X-C (VCX-C), mRNA.
ARRDC4	Homo sapiens arrestin domain containing 4 (ARRDC4), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
GDF15	Homo sapiens growth differentiation factor 15 (GDF15), mRNA.
RAET1L	Homo sapiens retinoic acid early transcript 1L (RAET1L), mRNA.
BGN	Homo sapiens biglycan (BGN), mRNA.
NEFM	Homo sapiens neurofilament, medium polypeptide 150kDa (NEFM), mRNA.
KRT6A	Homo sapiens keratin 6A (KRT6A), mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA
RDM1	Homo sapiens RAD52 motif 1 (RDM1), transcript variant 2, mRNA.
VCX3A .	Homo sapiens variable charge, X-linked 3A (VCX3A), mRNA
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.
C17orf91	Homo sapiens chromosome 17 open reading frame 91 (C17orf91), transcript variant 2, mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1, mRNA.
DKK3	Homo sapiens dickkopf homolog 3 (Xenopus laevis) (DKK3), transcript variant 2, mRNA.
FAM111A	Homo sapiens family with sequence similarity 111, member A (FAM111A), transcript variant 1, mRNA.
Genes differentially expressed after 21 Days of reprogramming	

GeneSymbol	Description
HBE1	Homo sapiens hemoglobin, epsilon 1 (HBE1), mRNA.
FILIP1	Homo sapiens filamin A interacting protein 1 (FILIP1), mRNA.
CCL20	Homo sapiens chemokine (C-C motif) ligand 20 (CCL20), mRNA.
MGC61598	PREDICTED: Homo sapiens similar to ankyrin-repeat protein Nrarp (MGC61598), mRNA.
LOC645682	PREDICTED: Homo sapiens hypothetical LOC645682 (LOC645682), mRNA.
TGFBI	Homo sapiens transforming growth factor, beta-induced, 68kDa (TGFBI), mRNA.
GAGE12G	Homo sapiens G antigen 12G (GAGE12G), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
T1560	Homo sapiens T1560 protein (T1560), mRNA.
HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
LIM2	Homo sapiens lens intrinsic membrane protein 2, 19kDa (LIM2), mRNA.
IGFBP6	Homo sapiens insulin-like growth factor binding protein 6 (IGFBP6), mRNA.
LOC644743	PREDICTED: Homo sapiens hypothetical LOC644743 (LOC644743), mRNA.
AKR1B10	Homo sapiens aldo-keto reductase family 1, member B10 (aldose reductase) (AKR1B10), mRNA.
ADAMTS1	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS1), mRNA.
TKTL1	Homo sapiens transketolase-like 1 (TKTL1), mRNA.
LOC645037	Homo sapiens similar to GAGE-2 protein (G antigen 2) (LOC645037), mRNA.
FLJ21986	Homo sapiens hypothetical protein FLJ21986 (FLJ21986), mRNA.
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
LOC387763	PREDICTED: Homo sapiens hypothetical LOC387763 (LOC387763), mRNA.
DPYSL3	Homo sapiens dihydropyrimidinase-like 3 (DPYSL3), mRNA.
ZCCHC12	Homo sapiens zinc finger, CCHC domain containing 12 (ZCCHC12), mRNA.
LOC643272	PREDICTED: Homo sapiens hypothetical protein LOC643272 (LOC643272), mRNA.
ELSPBP1	PREDICTED: Homo sapiens epididymal sperm binding protein 1 (ELSPBP1), mRNA.
LOC404266	PREDICTED: Homo sapiens hypothetical LOC404266 (LOC404266), misc RNA.
S100A4	Homo sapiens S100 calcium binding protein A4 (S100A4), transcript variant 2, mRNA.
VCX	Homo sapiens variable charge, X-linked (VCX), mRNA.
CRYBA4	Homo sapiens crystallin, beta A4 (CRYBA4), mRNA.
ETS1	Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), mRNA.
MAGEB2	Homo sapiens melanoma antigen family B, 2 (MAGEB2), mRNA.
IL13RA2	Homo sapiens interleukin 13 receptor, alpha 2 (IL13RA2), mRNA.
S100A4	Homo sapiens S100 calcium binding protein A4 (S100A4), transcript variant 2, mRNA.
C21orf34	Homo sapiens chromosome 21 open reading frame 34 (C21orf34), transcript variant 3, mRNA.
ZNF342	Homo sapiens zinc finger protein 342 (ZNF342), mRNA.
FES	Homo sapiens feline sarcoma oncogene (FES), mRNA.

LMNA	Homo sapiens lamin A/C (LMNA), transcript variant 2, mRNA.
DCT	Homo sapiens dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2), mRNA.
C21orf34	Homo sapiens chromosome 21 open reading frame 34 (C21orf34), transcript variant 1, mRNA.
LOC646817	PREDICTED: Homo sapiens similar to Protein SET (LOC646817), mRNA.
POU5F1	Homo sapiens POU class 5 homeobox 1 (POU5F1), transcript variant 1, mRNA.
VCX2	Homo sapiens variable charge, X-linked 2 (VCX2), mRNA.
LOC648526	PREDICTED: Homo sapiens similar to epiplakin 1 (LOC648526), mRNA.
IRS4	Homo sapiens insulin receptor substrate 4 (IRS4), mRNA.
S100A6	Homo sapiens S100 calcium binding protein A6 (S100A6), mRNA.
LSS	Homo sapiens lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) (LSS), transcript variant 1, mRNA.
TNC	Homo sapiens tenascin C (hexabrachion) (TNC), mRNA.
GCGR	Homo sapiens glucagon receptor (GCGR), mRNA.
MMP1	Homo sapiens matrix metallopeptidase 1 (interstitial collagenase) (MMP1), mRNA.
S100A16	Homo sapiens S100 calcium binding protein A16 (S100A16), mRNA.
BCL6	Homo sapiens B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6), transcript variant 1, mRNA.
GAGE5	Homo sapiens G antigen 5 (GAGE5), mRNA.
OLFM1	Homo sapiens olfactomedin 1 (OLFM1), transcript variant 1, mRNA.
KCTD12	Homo sapiens potassium channel tetramerisation domain containing 12 (KCTD12), mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 1, mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (TEK), mRNA.
SAT1	Homo sapiens spermidine/spermine N1-acetyltransferase 1 (SAT1), mRNA.
KRT8	Homo sapiens keratin 8 (KRT8), mRNA.
SPRYD5	Homo sapiens SPRY domain containing 5 (SPRYD5), mRNA.
TRIM7	Homo sapiens tripartite motif-containing 7 (TRIM7), transcript variant 6, mRNA.
STC1	Homo sapiens stanniocalcin 1 (STC1), mRNA.
KIF1A	Homo sapiens kinesin family member 1A (KIF1A), mRNA.
CALB1	Homo sapiens calbindin 1, 28kDa (CALB1), mRNA.
EGR1	Homo sapiens early growth response 1 (EGR1), mRNA.
MAFB	Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) (MAFB), mRNA.
D4S234E	Homo sapiens DNA segment on chromosome 4 (unique) 234 expressed sequence (D4S234E), trans 2, mRNA.
COL3A1	Homo sapiens collagen, type III, a 1 (Ehlers-Danlos syndrome type IV, autosomal dominant) (COL3A1), mRNA.
TSPYL2	Homo sapiens TSPY-like 2 (TSPYL2), mRNA.
IL1B	Homo sapiens interleukin 1, beta (IL1B), mRNA.
TRIM22	Homo sapiens tripartite motif-containing 22 (TRIM22), mRNA.
FAM133A	Homo sapiens family with sequence similarity 133, member A (FAM133A),

	mRNA.
GAGE5	Homo sapiens G antigen 5 (GAGE5), mRNA.
SOX21	Homo sapiens SRY (sex determining region Y)-box 21 (SOX21), mRNA.
GAGE6	Homo sapiens G antigen 6 (GAGE6), mRNA.
MMP3	Homo sapiens matrix metallopeptidase 3 (stromelysin 1, progelatinase) (MMP3), mRNA.
COL1A2	Homo sapiens collagen, type I, alpha 2 (COL1A2), mRNA.
NOV	Homo sapiens nephroblastoma overexpressed gene (NOV), mRNA.
ZNF503	Homo sapiens zinc finger protein 503 (ZNF503), mRNA.
CRMP1	Homo sapiens collapsin response mediator protein 1 (CRMP1), transcript variant 1, mRNA.
PRRX2	Homo sapiens paired related homeobox 2 (PRRX2), mRNA.
CDH10	Homo sapiens cadherin 10, type 2 (T2-cadherin) (CDH10), mRNA.
HSPB8	Homo sapiens heat shock 22kDa protein 8 (HSPB8), mRNA.
INSIG1	Homo sapiens insulin induced gene 1 (INSIG1), transcript variant 2, mRNA.
HES5	Homo sapiens hairy and enhancer of split 5 (Drosophila) (HES5), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.
INSM1	Homo sapiens insulinoma-associated 1 (INSM1), mRNA.
S100P	Homo sapiens S100 calcium binding protein P (S100P), mRNA.
CPLX1	Homo sapiens complexin 1 (CPLX1), mRNA.
ZNF280A	Homo sapiens zinc finger protein 280A (ZNF280A), mRNA.
CD44	Homo sapiens CD44 molecule (Indian blood group) (CD44), transcript variant 4, mRNA.
HCP5	Homo sapiens HLA complex P5 (HCP5), mRNA.
HSPA6	Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA.
	qk01b06.x1 NCI_CGAP_Kid3 Homo sapiens cDNA clone IMAGE:1867667 3, mRNA sequence
	EST10539 Adipose tissue, white I Homo sapiens cDNA 5 end, mRNA sequence
	yu38g09.r1 Soares ovary tumor NbHOT Homo sapiens cDNA clone IMAGE:236128 5, mRNA sequence
SNORD13	Homo sapiens small nucleolar RNA, C/D box 13 (SNORD13) on chromosome 8.
	UI-E-EJ1-ajw-b-16-0-UI.r1 UI-E-EJ1 Homo sapiens cDNA clone UI-E-EJ1-ajw-b-16-0-UI 5, mRNA sequence
IFI27	Homo sapiens interferon, alpha-inducible protein 27 (IFI27), mRNA.
SYK	Homo sapiens spleen tyrosine kinase (SYK), mRNA.
SCG5	Homo sapiens secretogranin V (7B2 protein) (SCG5), mRNA.
TEK	Homo sapiens TEK tyrosine kinase, endothelial (TEK), mRNA.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
POU5F1P1	Homo sapiens POU class 5 homeobox 1 pseudogene 1 (POU5F1P1) on chromosome 8.
ITM2A	Homo sapiens integral membrane protein 2A (ITM2A), mRNA.
MAGEC2	Homo sapiens melanoma antigen family C, 2 (MAGEC2), mRNA.
HES2	Homo sapiens hairy and enhancer of split 2 (Drosophila) (HES2), mRNA.
COL1A2	Homo sapiens collagen, type I, alpha 2 (COL1A2), mRNA.
CALCB	Homo sapiens calcitonin-related polypeptide beta (CALCB), mRNA.
STMN2	Homo sapiens stathmin-like 2 (STMN2), mRNA.

HBA2	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
SERPINB2	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), mRNA.
SERPINB2	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), mRNA.
CCNA1	Homo sapiens cyclin A1 (CCNA1), mRNA.
VCX-C	Homo sapiens variably charged X-C (VCX-C), mRNA.
IL8	Homo sapiens interleukin 8 (IL8), mRNA.
GDF15	Homo sapiens growth differentiation factor 15 (GDF15), mRNA.
FLVCR2	Homo sapiens feline leukemia virus subgroup C cellular receptor family, member 2 (FLVCR2), mRNA.
RAET1L	Homo sapiens retinoic acid early transcript 1L (RAET1L), mRNA.
NEFM	Homo sapiens neurofilament, medium polypeptide 150kDa (NEFM), mRNA.
KRT6A	Homo sapiens keratin 6A (KRT6A), mRNA.
GAGE12I	Homo sapiens G antigen 12I (GAGE12I), mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.
CXorf48	Homo sapiens chromosome X open reading frame 48 (CXorf48), transcript variant 1, mRNA.
DCN	Homo sapiens decorin (DCN), transcript variant C, mRNA.
VCX3A	Homo sapiens variable charge, X-linked 3A (VCX3A), mRNA.
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 2, mRNA.
IL32	Homo sapiens interleukin 32 (IL32), transcript variant 4, mRNA.
TSC22D3	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.
C17orf91	Homo sapiens chromosome 17 open reading frame 91 (C17orf91), transcript variant 2, mRNA.
IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1, mRNA.
DNMT3L	Homo sapiens DNA (cytosine-5-)-methyltransferase 3-like (DNMT3L), transcript variant 1, mRNA.
FAM111A	Homo sapiens family with sequence similarity 111, member A (FAM111A), transcript variant 1, mRNA.

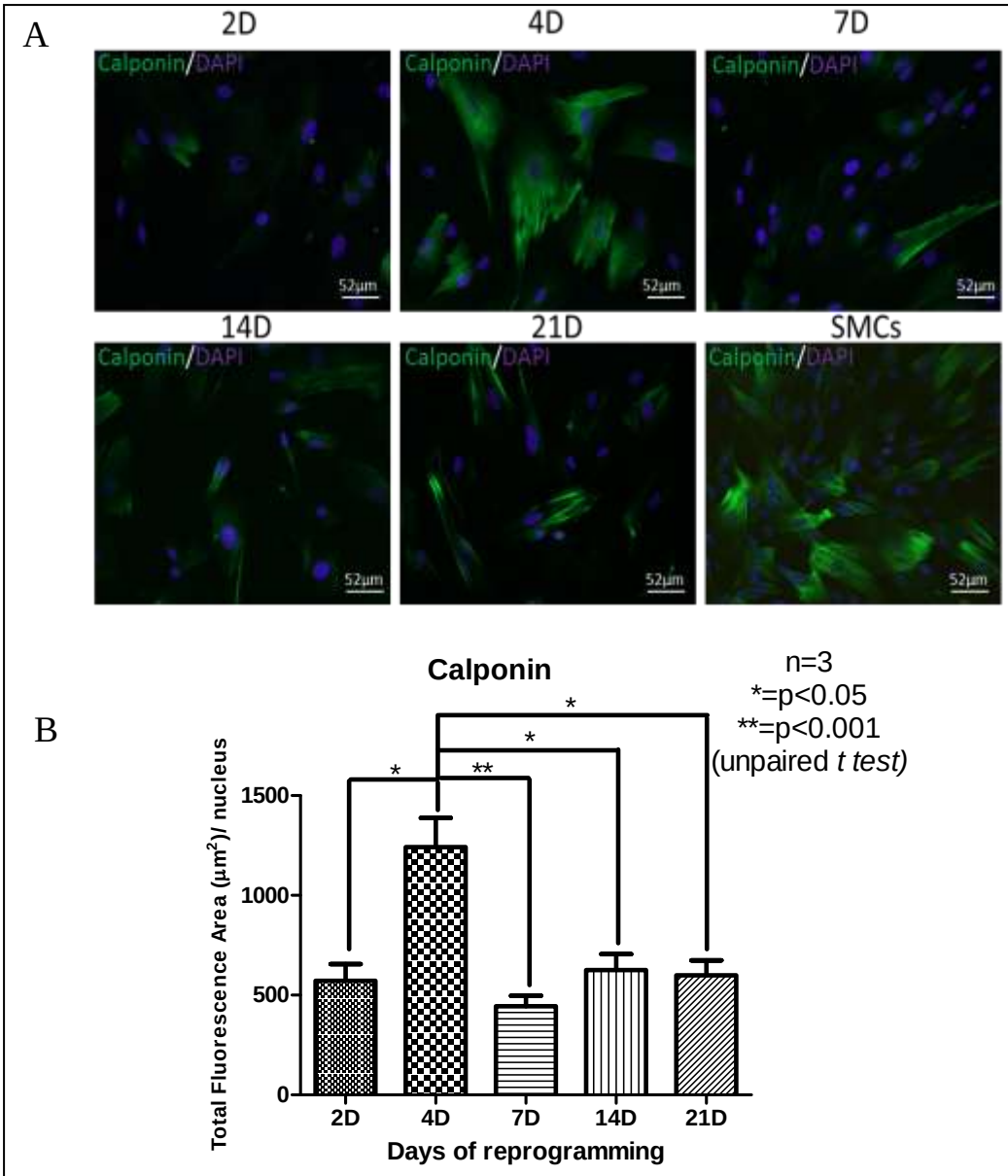


Figure S2. **Confirmation of optimal timepoint of reprogramming for SMC differentiation. (A)** Fibroblasts were reprogrammed for 2, 4, 7 14 and 21 days and then differentiated for 4 days. Indirect immunoflueorescence for Calponin was then performed as indication of SMC differentiation. DAPI was used as counterstaining for identification of the nucleus. Images were obtained with the Olympus IX81 white field inverted microscope. **(B)** Quantification of Calponin expression in differentiated cells using the Volocity Imaging software.

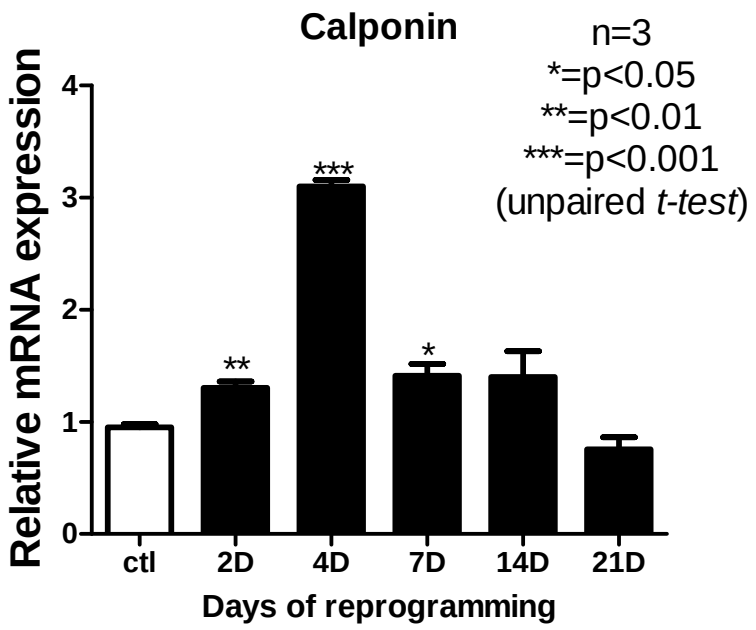
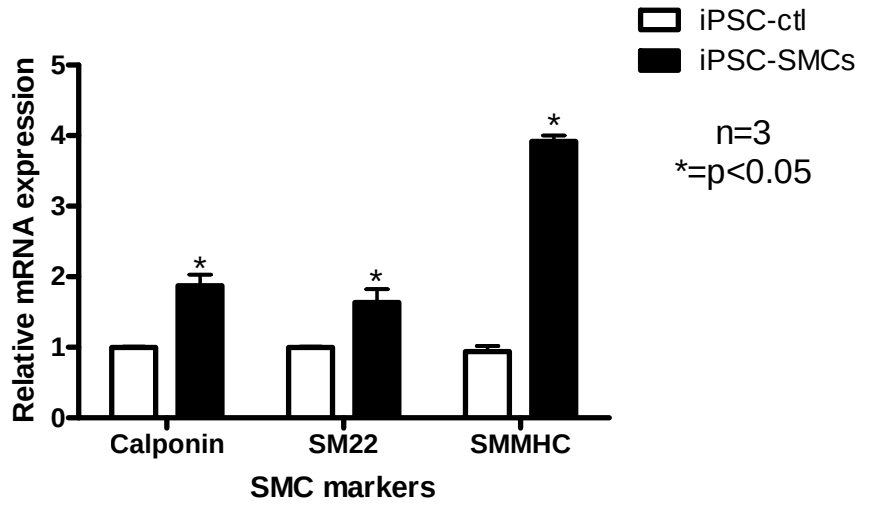


Figure S3. Confirmation of optimal day of reprogramming for SMC differentiation on the mRNA level. Fibroblasts were reprogrammed for 2, 4, 7, 14 and 21 days and then differentiated for 4 days. Non reprogrammed fibroblasts were used as a ctl. Total mRNA was extracted and Q-PCR was performed for Calponin (the mean ±SEM of 3 samples is shown).

A



B

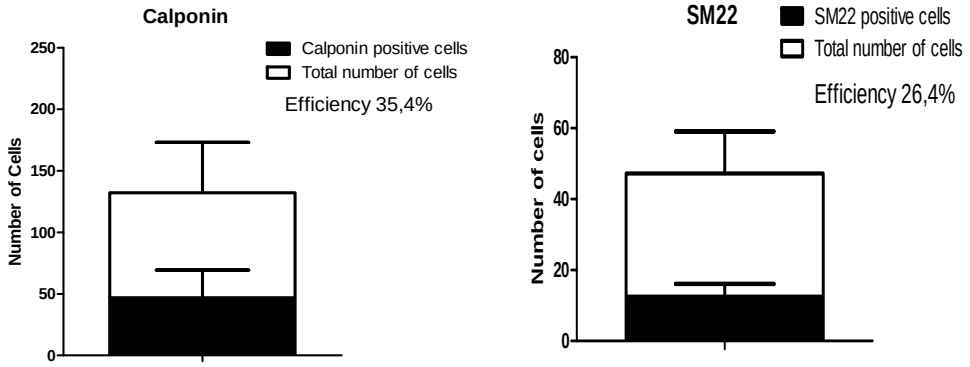
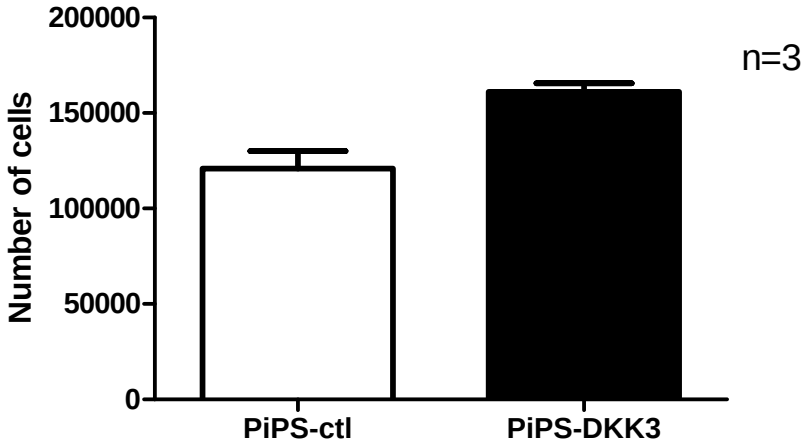


Figure S4. (A) Expression of SMC markers in differentiated iPS cells. Human iPS cells were cultured on Collagen IV and DM for 6 days. Samples were then harvested and subjected to Q-PCR. Undifferentiated iPS cells were used as a ctl (mean ±SEM of 3 individual experiments). **(B) Overall efficiency of differentiation per culture.** PiPS cells were differentiated on Collagen IV for 4 days and then stained for SMC markers Calponin and SM22. The cells which stained positive for the aforementioned markers were quantified and compared to the total amount of cells per culture (mean ±SEM of 3 individual experiments).

A



B

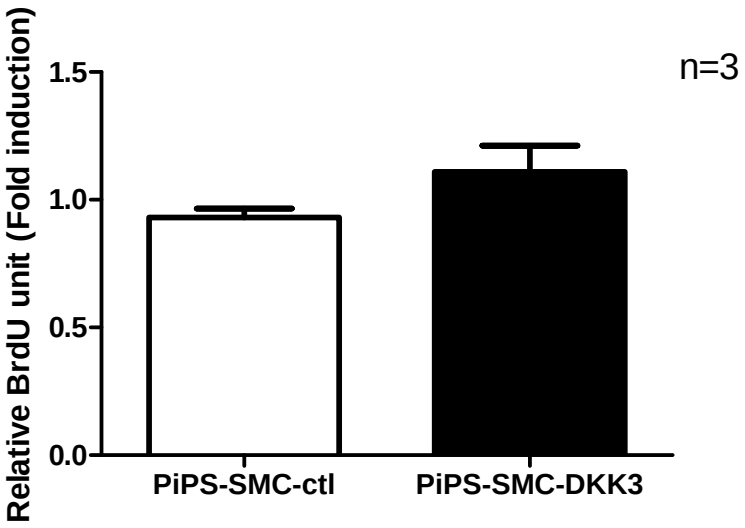


Figure S5. Overexpression of DKK3 does not affect the proliferation rate of PiPS-SMCs. PiPS-SMCs were differentiated on Collagen IV and DM for 2 days and then nucleofected with an empty PCMV5 vector or a DKK3 vector. Cells were then trypsinised and counted **(A)** or subjected to a BrdU Elisa assay **(B)** to assess proliferation rates (mean ±SEM of 3 individual samples).

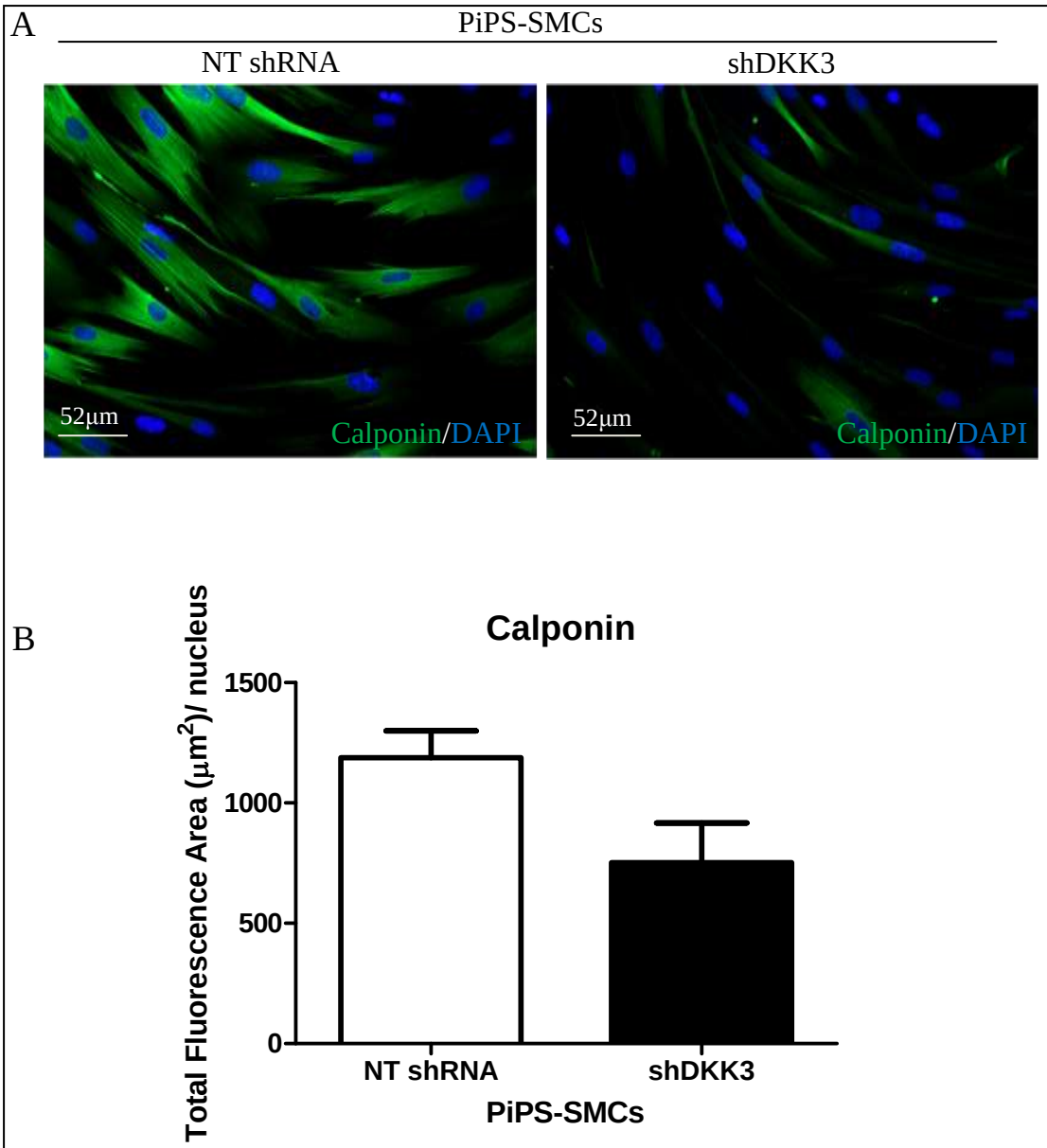


Figure S6. PiPS-SMCs present a reduced differentiation potential after lentiviral silencing of DKK3. PiPS cells were seeded on Collagen IV and DM and then infected with NT shRNA or shDKK3 for 72 hours. Indirect immunofluorescence for Calponin reveals that there is a reduced differentiation potential of PiPS to SMCs after 72 hours of DKK3 silencing. DAPI was used as counterstaining for identification of the nucleus. Images were obtained with the Olympus IX81 white field inverted microscope. **(B)** Quantification of Calponin expression in differentiated cells using the Volocity Imaging software.

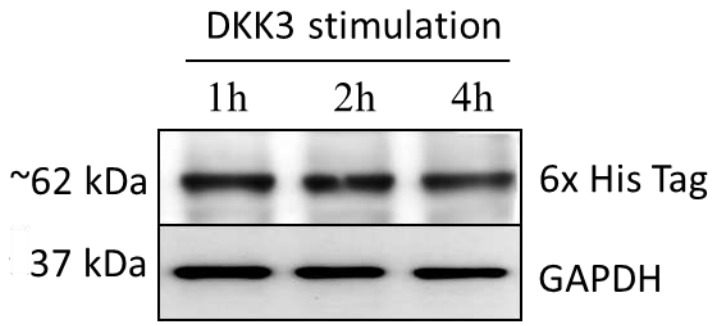


Figure S7. Confirmation of activity of human recombinant DKK3 protein. Fibroblasts were stimulated for different timepoints with human recombinant DKK3 tagged with 6xHis Tag. Samples were harvested and subjected to western blot analysis for 6xHis Tag revealing that the recombinant protein is successfully received by fibroblasts after 2 hours of stimulation.

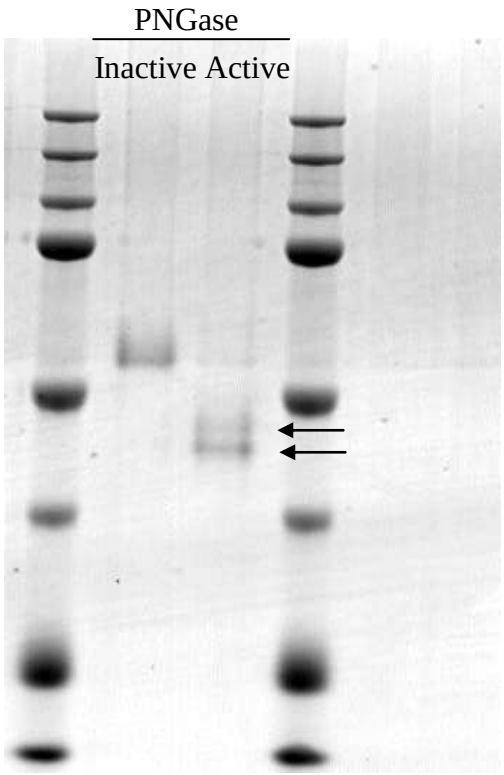


Figure S8. Confirmation of successful DKK3 deglycosylation. Human recombinant DKK3 was deglycosylated with heat inactivated PNGase (boiled at 96^{o}C) and active PNGase for 5 hours. The samples were then subjected to western blot analysis for DKK3. The molecular weight of DKK3 was reduced under conditions of successful deglycosylation.

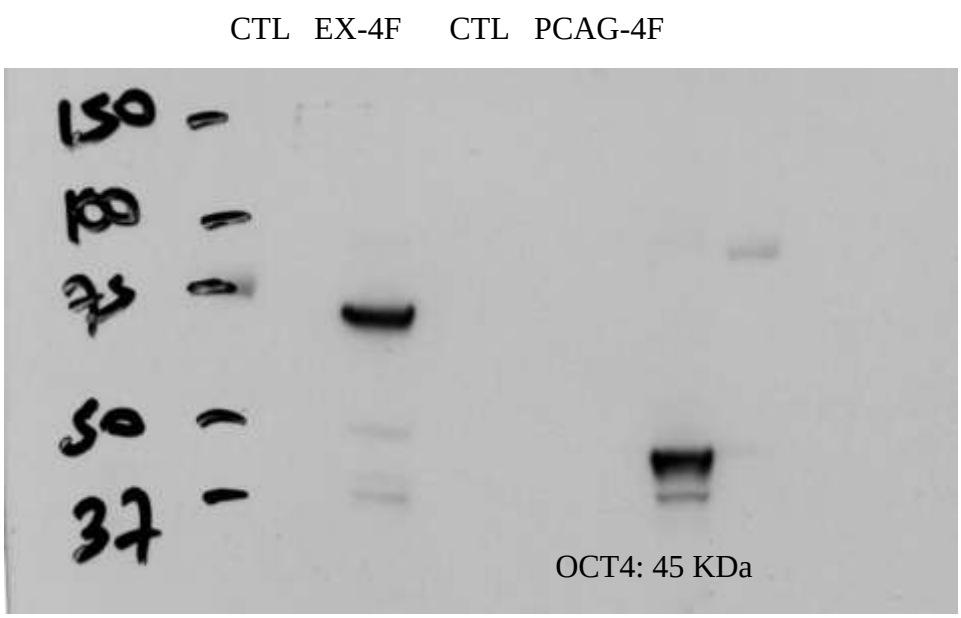


Figure S9. Representative image of antibody specificity. Fibroblasts were infected with lentiviruses carrying Ex-SOX2/Ex-OCT4/Ex-KLF4/ex-cMyc or nucleofected with the pCAG$_2$L-OSKM vector carrying all four factors. Fibroblasts were also infected/nucleofected with EX-GFP and ctl pCAG2L vector respectively. Samples were subjected to western blot analysis and were blotted for OCT4. The predicted molecular weight for OCT4 is 45 kDa. For the Ex-OCT4 infected samples, the specific band appears at size ~72 due to the GFP included in the gene sequence (molecular weight 27 kDa).

Chapter 6
Publications and Awards

Journal Articles

Published

Margariti A, Zampetaki A, Xiao Q, Zhou B, **Karamariti E,** Martin D, Yin X, Mayr M, Li H, Zhang Z, De Falco E, Hu Y, Cockerill G, Xu Q, Zeng L. **Histone deacetylase 7 controls endothelial cell growth through modulation of beta-catenin.** Circ Res. 2010 Apr 16;106(7):1202-11. Epub 2010 Mar 11.

Margariti A, Winkler B, Karamariti E, Zampetaki A, Tsai T, Baban D, Ragoussis J, Zeng L, Hu Y and Xu Q. Direct Reprogramming of Fibroblasts into Endothelial Cells Capable of Angiogenesis and Re-endothelisation in Tissue Engineered Vessels. *Proc Natl Acad Sci U S A*. 2012 (In press).

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A Margariti, B Winkler, **E Karamariti**, T Tsai, L Zeng, Y Hu, Q Xu. **Direct reprogramming fibroblasts into functional endothelial cells capable of vasculogenesis.** Stem Cell Programming & Reprogramming, Cell Symposia 8-10 December 2011 Lisbon, Portugal (Poster Presentation).

A Margariti, J P Kirton, X Yin, **E Karamariti**, M Mayr, Y Hu, L Zeng, Q Xu. **Proteomic analysis of iPS and embryonic stem cells identifies alternate cell differentiation properties.** Stem Cell Programming & Reprogramming, Cell Symposia 8-10 December 2011 Lisbon, Portugal (Poster Presentation).

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Chapter 7
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